Express Mail No. EV 334343630 US

CA IX-SPECIFIC INHIBITORS

By

Claudiu Supuran
Via de Cerchi 6
50122 Florence, Italy

Andrea Scozzafava
Via G. Segato 3
50134 Florence, Italy

Silvia Pastorekova
I. Bukovcana 18, 84107
Bratislava, Slovakia

Jaromir Pastorek
I. Bukovcana 18, 84107
Bratislava, Slovakia

CA IX-SPECIFIC INHIBITORS

This application claims priority from U.S. Provisional Application Nos. 60/429,089 (filed on November 26, 2002), 60/489,473 (filed on July 22, 2003) and 60/515,140 (filed on October 28, 2003).

FIELD OF THE INVENTION

The present invention is in the general area of medical genetics and in the fields of chemistry, biochemical engineering, and oncology. More specifically, it relates to the use of organic and inorganic compounds, preferably aromatic and heterocyclic sulfonamides, to treat preneoplastic and/or neoplastic diseases by specifically inhibiting the carbonic anhydrase activity of the oncoprotein now known alternatively as the MN protein, the MN/CA IX isoenzyme, the MN/G250 protein or simply MN/CA IX or CA IX or MN. The present invention also relates to methods of treating preneoplastic and/or neoplastic diseases characterized by MN/CA IX overexpression by administering cell membrane-impermeant, inhibitors of MN/CA IX, preferably pyridinium derivatives of aromatic and heterocyclic sulfonamides. The invention further concerns diagnostic/prognostic methods including imaging methods, for preneoplastic/neoplastic diseases, using the disclosed potent CA IX-specific inhibitors, and gene therapy with vectors conjugated to said inhibitors.

BACKGROUND OF THE INVENTION

The instant inventors, Dr. Silvia Pastorekova and Dr. Jaromir Pastorek, with Dr. Jan Zavada ["Zavada et al."], discovered MN/CA IX, a cancer related cell surface protein originally named MN. [*73, 123;* Zavada et al., U.S. Patent No. 5,387,676 (Feb. 7,1995).] Zavada et al., WO 93/18152 (published 16 September 1993) and Zavada et al., WO 95/34650 (published 21 December 1995) disclosed the discovery of the MN gene and protein and the strong association of MN gene expression and tumorigenicity led to the creation of methods that are both diagnostic/prognostic and therapeutic for cancer and precancerous conditions.

Zavada et al. disclosed further aspects of the MN/CA IX protein and the MN/CA9 gene in Zavada et al., WO 00/24913 (published 4 May 2000).

Zavada et al. cloned and sequenced the MN cDNA and gene, and revealed that MN belongs to a carbonic anhydrase family of enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and proton [*66*, *72*]. MN protein (renamed to carbonic anhydrase IX, CA IX) is composed of an extracellular part containing a N-terminal proteoglycan-like region and a catalytically active carbonic anhydrase domain. It is anchored in the plasma membrane by a single transmembrane region and a short intracytoplasmic tail.

Expression of CA IX is restricted to only few normal tissues [*74*], but is tightly associated with tumors [*123*]. It is also regulated by cell density *in vitro* [*52*] and is strongly induced by tumor hypoxia both *in vitro* and *in vivo* [*121*]. Numerous clinical papers describe the value of CA IX as an indicator of poor prognosis. All CA IX-related studies performed so far support the assumption made in the original Zavada et al., U.S Patent 5,387,676 that CA IX is useful as a diagnostic and/or prognostic tumor marker and as a therapeutic target.

MN/CA IX consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail [*66*, *72, 74, 116*]. CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, ovarian, kidney, lung, esophagus, breast, colon, endometrial, bladder, colorectal, prostate, among many other human carcinomas, contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract [*8, 11, 21, 35, 41, 48, 50, 51, 56, 66, 72, 74, 86, 110, 111, 113, 116, 121, 122*].

Uemura et al. [*112*] reported in 1997 that the G250 antigen was identical to MN/CA IX, years after MN/CA IX had been discovered and sequenced by Zavada et al. {[*73, 123*]; see also Pastorek et al. [*72]* and Opavsky et al. [*66*]}. Uemura et al. [*112*] stated: "Sequence analysis and database searching revealed that G250 antigen is identical to MN a human tumor-associated antigen identified in cervical carcinoma (Pastorek et al., 1994)."

MN/CA 9 and MN/CA IX – Sequence Similarities

Figure 1A-C shows the full-length MN/CA9 cDNA sequence of 1522 base pairs (bps) [SEQ ID NO: 1], and the full-length MN/CA IX amino acid (aa) sequence of 459 aa [SEQ ID NO: 2]. Figure 2A-F provides the 10,898 bp genomic sequence of MN/CA9 [SEQ ID NO: 3].

Computer analysis of the MN cDNA sequence was carried out using DNASIS and PROSIS (Pharmacia Software packages). GenBank, EMBL, Protein Identification Resource and SWISS-PROT databases were searched for all possible sequence similarities. In addition, a search for proteins sharing sequence similarities with MN was performed in the MIPS databank with the FastA program [*75*].

The proteoglycan-like domain [aa 53-111; SEQ ID NO: 4] which is between the signal peptide and the CA domain, shows significant homology (38% identity and 44% positivity) with a keratan sulphate attachment domain of a human large aggregating proteoglycan aggrecan [*28*].

The CA domain [aa 135-391; SEQ ID NO: 5] is spread over 265 aa and shows 38.9% amino acid identity with the human CA VI isoenzyme [*5*]. The homology between MN/CA IX and other isoenzymes is as follows: 35.2% with CA II in a 261 aa overlap [*63*], 31.8% with CA I in a 261 aa overlap [*7*], 31.6% with CA IV in a 266 aa overlap [*65*], and 30.5% with CA III in a 259 aa overlap [*55*].

In addition to the CA domain, MN/CA IX has acquired both N-terminal and C-terminal extensions that are unrelated to the other CA isoenzymes. The amino acid sequence of the C-terminal part, consisting of the transmembrane anchor and the intracytoplasmic tail, shows no significant homology to any known protein sequence.

The MN gene (MN/CA9 or CA9) was clearly found to be a novel sequence derived from the human genome. The overall sequence homology between the cDNA MN/CA9 sequence and cDNA sequences encoding different CA isoenzymes is in a homology range of 48-50% which is considered by ones in the art to be low. Therefore, the MN/CA9 cDNA sequence is not closely related to any CA cDNA sequences.

Very few normal tissues have been found to express MN protein to any significant degree. Those MN-expressing normal tissues include the human gastric

mucosa and gallbladder epithelium, and some other normal tissues of the alimentary tract. Paradoxically, MN gene expression has been found to be lost or reduced in carcinomas and other preneoplastic/neoplastic diseases in some tissues that normally express MN, e.g., gastric mucosa.

CA IX and Hypoxia

Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of extent of necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC) [*8, 11, 21, 35, 48, 56, 111, 122*]. Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell-cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome [*8*]. Hypoxia is linked with acidification of extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity [*86*]. Thus, inhibition of MN/CA IX by specific inhibitors is considered to constitute a novel approach to the treatment of cancers in which CA IX is expressed.

CAIs

Teicher et al. [*106*] reported that acetazolamide – the prototypical CA inhibitor (CAI) – functions as a modulator in anticancer therapies, in combination with different cytotoxic agents, such as alkylating agents; nucleoside analogs; platinum derivatives, among other such agents, to suppress tumor metastasis and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498). Such studies demonstrate that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It was hypothesized that the anticancer effects of acetazolamide (alone or in combination with such drugs) might be due to the acidification of the intratumoral environment ensuing after CA inhibition, although other mechanisms of action of this drug were not excluded [*20*]. Chegwidden et al. 2001 hypothesized that the *in vitro* inhibition of growth in cell cultures, of human lymphoma cells with two other potent, clinically used sulfonamide

CAIs, methazolamide and ethoxzolamide, is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition [*20*].

All the six classical CAIs (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and dichlorophenamide) used in clinical medicine or as diagnostic tools, show some tumor growth inhibitory properties [*18, 78, 101, 102*].

The inventors, Dr. Claudia Supuran and Dr. Andrea Scozzafava, reported the design and *in vitro* antitumor activity of several classes of sulfonamide CAIs, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II and CA IV. Those compounds were also shown to exert potent inhibition of cell growth in several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines, with GI_{50} values of 10 – 75 nM in some cases [*77, 91, 92, 100*].

Wingo et al. reported that three classic sulfonamide drugs (acetozolamide, ethoxzolamide and methoxzolamide) inhibited CA IX carbonic anhydrase activity with values of K_I in the nanomolar range [*116*]. However, until the present invention, no systematic structure-activity relationship study of sulfonamide inhibition of CA IX, alone or in comparison to other CA isozymes had been been performed.

Certain pyridinium derivatives of aromatic/heterocyclic sulfonamides have shown nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes *in vivo* [*17*].

Sterling et al. [*85*] investigated the functional and physical relationship between the downregulated in adenoma bicarbonate transporter and CA II, by using membrane-impermeant sulfonamide inhibitors (in addition to the classical inhibitors such as acetazolamide), which could clearly discriminate between the contribution of the cytosolic and membrane-associated isozymes in these physiological processes.

CAS

Carbonic anhydrases (CAs) form a large family of genes encoding zinc metalloenzymes of great physiological importance. As catalysts of reversible hydration of carbon dioxide, these enzymes participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, formation of aqueous humor, cerebrospinal fluid, saliva and gastric acid [reviewed in Dodgson et al. (*27*)]. CAs are widely distributed in different living organisms. In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described, with very different subcellular localization and tissue distribution [*40, 93, 95, 94, 102*]. Basically, there are several cytosolic forms (CA I-III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI [*40, 93, 94, 95, 102*].

It has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII [*2, 67, 68, 78, 87, 93 , 95*]. Occasionally, nuclear localization of some isoenzymes has been noted [*64, 69, 70*]. Not much is presently known about the cellular localization of the other isozymes.

CAs and CA-related proteins show extensive diversity in their tissue distribution, levels, and putative or established biological functions [*105*]. Some of the CAs are expressed in almost all tissues (CA II), while the expression of others appears to be more restricted (e.g., CA VI and CA VII in salivary glands [*32, 69, 71*]. The CAs and CA-related proteins also differ in kinetic properties and susceptibility to inhibitors [*82*].

Most of the clinically used sulfonamides mentioned above are systemically acting inhibitors showing several undesired side effects due to inhibition of many of the different CA isozymes present in the target tissue/organ (14 isoforms are presently known in humans) [*93, 94, 95, 102*]. Therefore, many attempts to design and synthesize new sulfonamides were recently reported, in order to avoid such side effects [*13, 17, 42, 62, 80, 99, 100*]. At least four CA isozymes (CA IV, CA IX, CA XII and CA XIV) are associated to cell membranes, with the enzyme active site generally oriented extracellularly [*93, 94, 95, 102*]. Some of these isozymes

were shown to play pivotal physiological roles (such as for example CA IV and XII in the eye, lungs and kidneys, CA IX in the gastric mucosa and many tumor cells) [3, 18, 22, 29, 49, 67, 68, 83, 93, 94, 95, 102], whereas the function of other such isozymes (CA XIV) is for the moment less well understood [*93, 95*]. Due to the extracellular location of these isozymes, if membrane-impermeant CA inhibitors (CAIs) could be designed, only membrane-associated CAs would be affected.

The first approach towards introducing the membrane-impermeability to CAIs from the historical point of view was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycol, aminoethyldextran, or dextran [*39, 60, 107*]. Such compounds, possessing molecular weights in the range of 3.5 - 99 kDa, prepared in that way, showed indeed membrane-impermeability due to their high molecular weights, and selectively inhibited *in vivo* only CA IV and not the cytosolic isozymes (primarily CA II), being used in several renal and pulmonary physiological studies [*39, 60, 107*]. Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since *in vivo* they induced potent allergic reactions [*39, 60, 93, 95, 107*]. A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has until recently been used in physiological studies, QAS (quaternary ammonium sulphanilamide), which has been reported to inhibit only extracellular CAs in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish [*57*]. The main draw-back of QAS is its high toxicity in higher vertebrates [*57*].

Enzyme activity of carbonic anhydrases (including that of CA IX) can be efficiently blocked by sulfonamide inhibitors. That fact has been therapeutically exploited in diseases caused by excessive activities of certain CA isoforms (e.g. CA II in glaucoma). There is also an experimental evidence that sulfonamides may block tumor cell proliferation and invasion *in vitro* and tumor growth *in vivo*, but the targets of those sulfonamides have not been identified yet. However, the sulfonamides available so far indiscriminately inhibit various CA isoenzymes (14 are presently known in humans) that are localized in different subcellular compartments and play diverse biological roles. This lack of selectivity compromises the clinical utilization of these compounds (due to undesired side effects caused by concurrent inhibition of

many CA isoforms) and represents a main drawback also for the sulfonamide application against CA IX in anticancer therapy.

Thus, there is a need in the art for membrane-impermeant, potent CA IX inhibitors, which would become doubly selective inhibitors for CA IX. The inventors have previously made and described some of the membrane-impermeant molecules described here; however, they were characterized only for their ability to inhibit CA I, CA II and CA IV. While others have studied effects of selective inhibition of extracellular CA by membrane impermeant agents in retinal prigmented epithelia or muscle [*34, 120*], these agents have not been characterized for their ability to inhibit CA IX. Since CA IX is one of the few extracellular carbonic anhydrases, a membrane-impermeant selective inhibitor of CA IX would be doubly selective for this enzyme and thereby avoid side effects associated with nonspecific CA inhibition.

SUMMARY OF THE INVENTION

The inventors approached the problem of lack of selectivity of CAIs by taking advantage of features that distinguish CA IX from the other CA isoforms. First of all, CA IX is an integral plasma membrane protein with an active site exposed on the extracellular side. In this respect, it is similar to some CAs (CA IV, CA XII and CA XIV) but differs from all other isoforms. Among these membrane-bound isoenzymes, CA IX shows some differences in the amino acid sequence of the catalytic domain that may influence the topology of the active site cavity and hence the interaction with sulfonamides. In addition, unlike the other CA isoforms, CA IX is expressed preferentially in hypoxic areas of tumors with poor prognosis.

The inventors evaluated inhibition profiles of CA IX with a series of aromatic and heterocyclic compounds and found that some of them inhibit CA IX more efficiently than the other widely distributed isoforms CA I, II and IV. Several nanomolar CA IX inhibitors have been detected both among the aromatic and the heterocyclic compounds. This finding is very promising for the design of CA IX-specific inhibitors by modification of their physico-chemical properties such as charge, size and bioreductivity to conform the characteristic properties of CA IX.

The inventors found that some of the more bulky compounds that strongly inhibited CA IX were very weak inhibitors of CA I, II and IV, possibly due to the fact that the CA IX active site cavity is larger than that of the other investigated isoenzymes. The compounds of such type, identified by screening as disclosed herein, based on the selective inhibition of tumor-associated isoform CA IX may be particularly preferred CA IX specific inhibitors, that could be used in new anticancer therapies and in the diagnostic/prognostic methods of this invention.

The inventors have shown that CA IX is capable of reducing E-cadherin-mediated cell-cell adhesion that may be important for increased invasion capacity of the cells [*103*]. CA IX was found by the inventors also to contribute to acidification of extracellular pH in hypoxia but not in normoxia *(unpublished data)*. The latter result indicates that hypoxia up-regulates both expression level and enzyme activity of CA IX, that is, hypoxia activates the CA catalytic activity of CA IX. That is a very important finding because intratumoral hypoxia is a clinically relevant factor increasing aggressiveness of tumor cells and reducing success of therapy. Hypoxia is usually accompanied by acidification of extracellular microenvironment, which facilitates tumor invasion and metastasis. CA IX appears to participate in this phenomenon by catalyzing hydration of carbon dioxide to generate bicarbonate ions that are then transported into cell interior and protons that acidify extracellular pH. Therefore, inhibition of the CA IX catalytic activity resulting in reduced extracellular acidification may have direct anticancer effects or may modulate efficiency of those conventional chemotherapeutic drugs whose uptake is pH-dependent.

The instant invention is related to (1) the recognition that certain carbonic anhydrase inhibitors (CAIs), preferably sulfonamides, selectively target the cancer-related, hypoxia-induced MN/CA IX; (2) the use of such CAIs, preferably sulfonamides, as lead compounds for the design and synthesis of MN/CA IX-specific inhibitors; (3) the employment of said MN/CA IX-specific inhibitors for anticancer therapy based upon the inhibition of MN/CA IX-mediated acidification of tumor microenvironments; and (4) the use of the specificity of potent MN/CA IX-specific inhibitors for diagnostic/prognostic methods including imaging methods, such as scintigraphy, and for gene therapy. The invention is particularly directed to the use of CA IX-specific inhibitors for the development of drugs possessing anticancer

properties and to modulate conventional chemotherapy for preneoplastic and neoplastic disease characterized by CA IX expression, particularly CA IX overexpression.

In one aspect, the invention concerns methods of treating a mammal for a pre-cancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprising administering to said mammal a therapeutically effective amount of a composition comprising a compound, wherein said compound is selected from the group consisting of organic and inorganic molecules, and wherein said compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising:

a) preparing serial dilutions of said compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;

b) preincubating a dilution of said compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;

c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO_2 solution, phenol red to 0.2mM, Na_2SO_4 to 0.1M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;

d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and

e) determining the inhibition constant K_I of said compound;

wherein if said inhibition constant K_I is determined to be less than about 50 nanomolar, said compound is determined be a potent inhibitor of MN/CA IX enzymatic activity; and wherein said compound is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate. Said mammal is preferably human, and said K_I is preferably less than about 35 nanomolar, more preferably less than about 25 nanomolar, and still more preferably less than about 10 nanomolar.

Such methods can also be framed as methods of treating precancer and/or cancer, or inhibiting the growth of precancerous and/or cancerous cells in a mammalian subject, wherein said precancer and cancer are characterized by the overexpression of MN/CA IX. Said methods can also be framed as inhibiting the growth of such precancerous or cancerous mammalian cells overexpressing MN/CA IX comprising contacting said cells with a CA IX-specific inhibitor of this invention.

The CA IX-specific inhibitors of this invention can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable nontoxic liquid vehicle. Different routes of administration may be preferred depending on the site or type of preneoplastic/neoplastic disease, for example, solid or non-solid tumor or metastasis. In general, parenteral administration would be preferred to avoid undesired effects of systemic treatment, for example, those that could be occasioned by binding of the inhibitors to the gastrointestinal mucosa. Injection into or into the vicinity of the preneoplastic/neoplastic disease would be generally preferred. For example, such injections could be intravenous, intraperitoneal, rectal, subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, intramedullary, intralesional, intradermal, among other routes of injection. Also, other modes of administration, for example, by suppository or topically, can be used as would be appropriate to the target disease. The pharmaceutical formulation would be designed in accordance with known standards as suitable for the route of administration.

Said CA IX-specific inhibitors are preferably organic, more preferably aromatic or heterocyclic, and still more preferably an aromatic sulfonamide or a heterocyclic sulfonamide. Said aromatic sulfonamide may be a substituted aromatic sulfonamide, wherein said aromatic sulfonamide comprises an aromatic ring structure bearing a sulfonamide moiety bonded to said ring structure and optionally bearing one or more substituents independently selected from the group consisting of halogeno, nitro, and an alkylamino group, wherein the alkyl radical of said alkylamino group comprises 1 to 4 carbon atoms.

Preferably the CA IX-specific inhibitors of this invention are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV.

More preferably, the CA IX-specific inhibitors are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Still more preferably, the CA IX-specific inhibitors are more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV.

However, since CA II is a particularly abundant and significant CA, that is cytosolic, it is important when the CA IX-specific inhibitors of this invention are not membrane-impermeant, that they may be more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of CA II. A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_I of a compound in inhibiting the enzymatic activity of CA II:

a) preparing serial dilutions of said compound and serial dilutions of CA II;

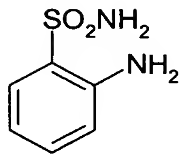
b) preincubating a dilution of said compound with a dilution of CA II for ten minutes at 20°C;

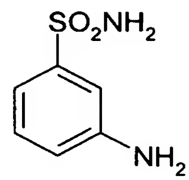
c) combining said preincubated mixture of said compound and said CA II with a substrate solution, consisting essentially of 4-nitrophenylacetate in anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;

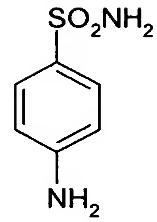
d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel, using a spectrophotometer; and

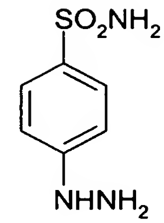
e) determining the inhibition constant K_I of said compound.

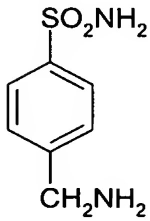
Exemplary and preferred aromatic sulfonamide or heterocyclic sulfonamide CA IX-specific inhibitors of this invention are selected from the group consisting of:

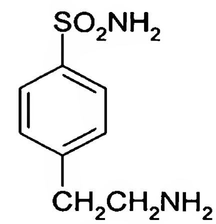

1

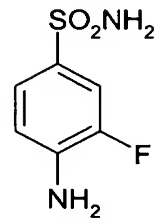

2

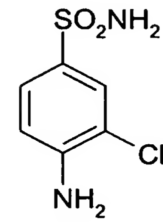

3

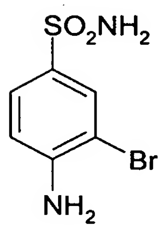

4

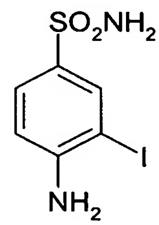

5

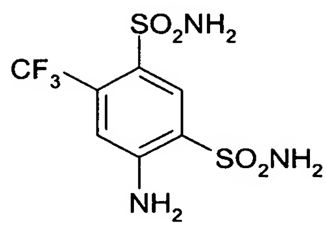

6

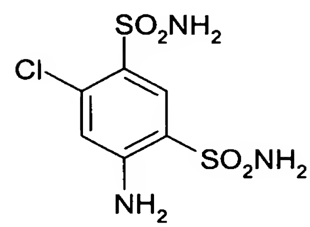

7

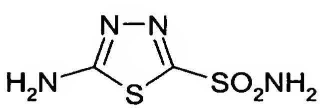

8

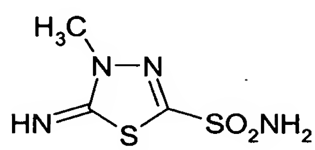

9

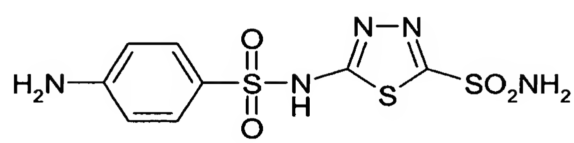

10

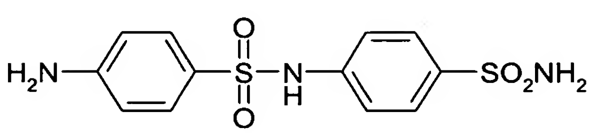

11

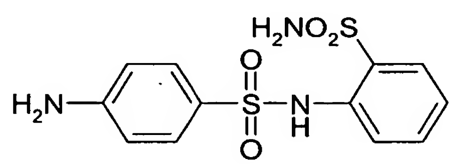

12

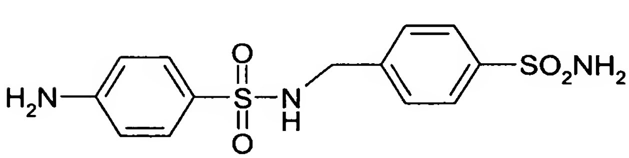

13

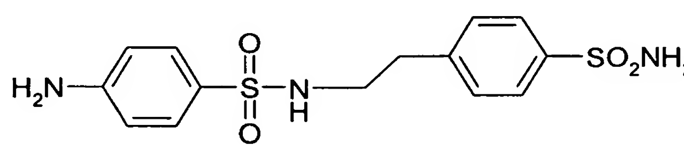

14

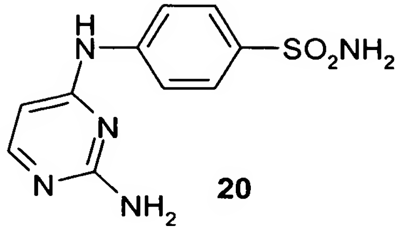

15


16

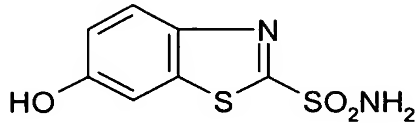

17


18

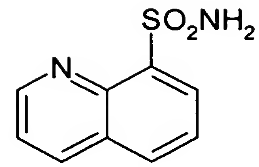

19



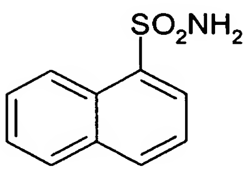
20



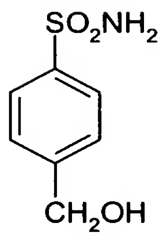
21



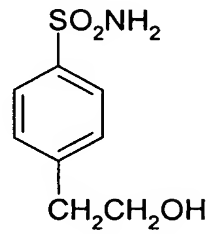
22



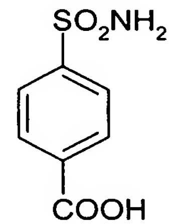
23



24

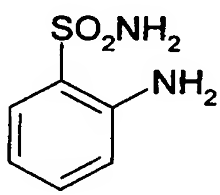


25

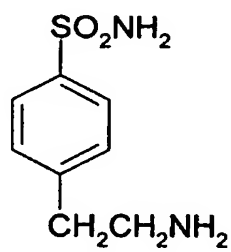


26

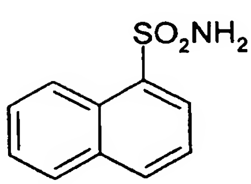
Exemplary preferred aromatic sulfonamide CA IX-specific inhibitors are selected from the group consisting of:



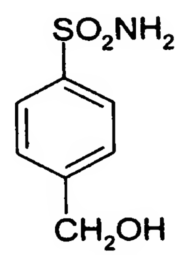
1 ,



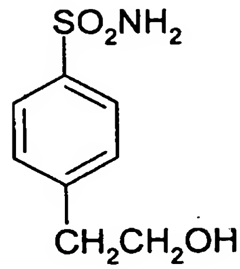
6 ,



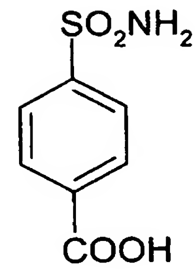
23 ,



24 ,



25

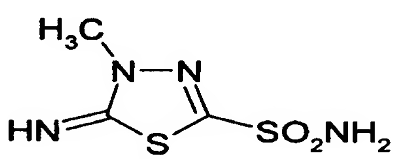


26

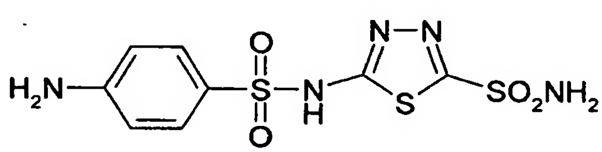
A preferred aromatic sulfonamide CA IX-specific inhibitor can be that wherein a halogen atom is bonded to at least one carbon atom in the aromatic ring of said aromatic sulfonamide.

Preferred heterocyclic sulfonamide CA IX-specific inhibitors can be substituted heterocyclic sulfonamides, wherein said substituted heterocyclic sulfonamide comprises a heterocyclic ring structure bearing a sulfonamide moiety bonded to said ring structure and optionally bearing one or more substituents independently selected from a group consisting of halogeno, nitro, and an alkylamino group, wherein the alkyl radical of said alkylamino group comprises 1 to 4 carbon atoms. Preferred heterocyclic sulfonamide CA IX-specific inhibitors may be halogenated.

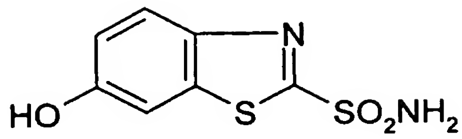
Further preferred heterocyclic sulfonamide CA IX-specific inhibitors are selected from the group consisting of:



14

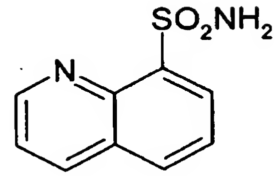


15



21

,



22

.

Further preferred methods of treating mammals for pre-cancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprise administering to said mammal membrane-impermeant CA IX-specific inhibitors. A therapeutically effective amount of such a membrane-impermeant CA IX-specific inhibitor can be administered in a composition comprising the membrane-impermeant compound, wherein said membrane-impermeant inhibitor compound is selected from the group consisting of organic and inorganic molecules, and wherein said membrane-impermeant compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising:

a) preparing serial dilutions of said membrane-impermeant compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;

b) preincubating a dilution of said membrane-impermeant compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;

c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO_2 solution, phenol red to 0.2mM, Na_2SO_4 to 0.1M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;

d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and

e) determining the inhibition constant K_I of said membrane-impermeant compound,

wherein if said inhibition constant K_I is determined to be less than about 50 nanomolar, said membrane-impermeant compound is determined be a potent inhibitor of MN/CA IX enzymatic activity. The mammal is preferably a human, and the K_I is preferably less than 35 nM, more preferably less than about 25 nM, and still more preferably less than about 10 nanomolar.

Such a membrane-impermeant CA IX specific inhibitor compound is preferably organic, and more preferably a pyridinium derivative of an aromatic sulfonamide or a pyridinium derivative of a heterocyclic sulfonamide. Such membrane-impermeant CA IX-specific inhibitor compounds are preferably more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV, and still more preferably more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Further more preferably, said membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV. Since both CA IX and CA IV are membrane bound CAs, it is particularly important that the membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of CA IV.

A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_I of a compound inhibiting the enzymatic activity of CA IV:

a) preparing serial dilutions of said membrane-impermeant compound and serial dilutions of CA IV;

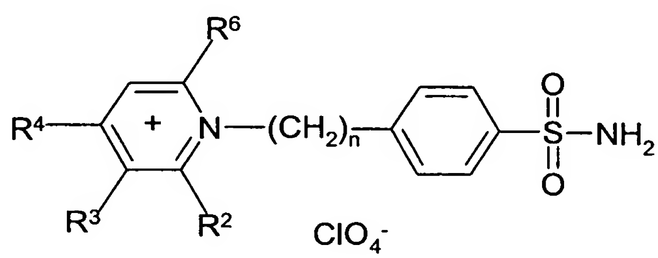
b) preincubating a dilution of said membrane-impermeant compound with a dilution of CA IV for ten minutes at 20°C;

c) combining said preincubated mixture of said compound and said CA IV with a substrate solution, consisting essentially of 4-nitrophenylacetate in anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;

d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel using a spectrophotometer; and

e) determining the inhibition constant K_I of said membrane-impermeant compound.

Preferred membrane-impermeant CA IX-specific inhibitor compounds that are pyridinium derivatives of aromatic sulfonamides are selected from the group consisting of sulfanilamide, homosulfanilamide and 4-aminoethyl-benzenesulfonamide. Preferred pyridinium derivatives of aromatic sulfonamides can have the general formula of:



wherein

n is 0, 1, or 2;

R2, R3, R4 and R6 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

R2 is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R3 is selected from the group consisting of hydrogen and methyl;

R4 is selected from the group consisting of hydrogen, methyl and phenyl; and

R6 is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, and phenyl. Still further preferred are such compounds wherein

R3 is hydrogen;

R4 and R6 are phenyl;

when n is 0, R2 is selected from the group consisting of methyl, ethyl, n-propyl, iso-propyl, n-butyl, and phenyl; and

when n is 1 or 2, R2 is selected from the group consisting of methyl, ethyl, n-propyl, iso-propyl, n-butyl, tert-butyl, and phenyl. Other preferred such compounds include those wherein

R3 is hydrogen;

R4 is phenyl; and

when n is 0, R2 and R6 are the same and are selected from the group consisting of methyl, ethyl, n-propyl, and iso-propyl; and

when n is 1 or 2, R2 and R6 are the same and are selected from the group consisting of methyl, ethyl, n-propyl and iso-propyl. Other preferred compounds include those wherein R2, R3, R4 and R6 are methyl. Still further preferred are such CA IX-specific inhibitor compounds wherein

when n is 0, 1 or 2, R2, R4 and R6 are methyl, and R3 is hydrogen; or

when n is 1 or 2, R2 is iso-propyl, R3 is hydrogen, R4 is methyl, and R6 is methyl or iso-propyl; or

when n is 1 or 2, R2 and R6 are phenyl, and R3 and R4 are hydrogen. Still more preferred such compounds are those wherein

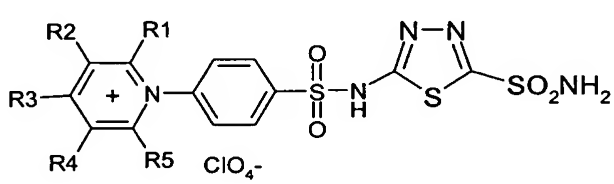
when n is 2, R2 and R6 are methyl, R3 is hydrogen, and R4 is phenyl; or

when n is 2, R2 and R6 are ethyl, R3 is hydrogen, and R4 is phenyl; or

when n is 2, R2, R3, R4 and R6 are methyl.

When said CA IX-specific inhibitors are membrane-impermeant pyridinium derivatives of a heterocyclic sulfonamides, a preferred compound is a pyridinium derivative of aminobenzolamide.

Preferred CA IX-specific inhibitor compounds that are pyridinium derivatives of heterocyclic sulfonamides may have the general formula of:



wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl and phenyl;

R2 is selected from the group consisting of hydrogen and methyl;

R3 is selected from the group consisting of hydrogen, methyl, *n*-nonyl, and phenyl;

R4 is selected from the group consisting of hydrogen and methyl; and

R5 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl, *n*-nonyl,and phenyl. Further preferred are such compounds wherein

R2 and R4 are hydrogen;

R3 is methyl; and

R1 and R5 are the same and selected from the group consisting of methyl, *iso*-propyl, and *tert*-butyl. Still further preferred are such compounds wherein

R2 and R4 are hydrogen;

R3 is phenyl; and

R1 and R5 are the same and selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, and phenyl. Additionally are preferred such compounds wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, and n-butyl;

R2 and R4 are hydrogen; and

R3 and R5 are phenyl. Other preferred such compounds are those wherein

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

R1 and R4 are methyl, R2 is hydrogen, and R3 and R5 are n-nonyl. Also preferred such compounds are those wherein

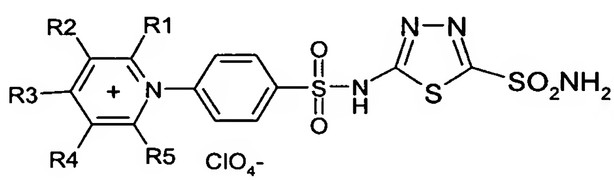
R1 is methyl or iso-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen; or

R1 and R5 are the same and are methyl or ethyl, R2 and R4 are hydrogen, and R3 is phenyl; or

R1, R2, R3 and R5 are methyl, and R4 is hydrogen.

In another aspect, this invention concerns methods of inhibiting tumor growth in a patient having a tumor, the cells of which tumor are characterized by overexpression of MN/CA IX protein, comprising administering to said patient a therapeutically effective amount of a composition comprising a compound, wherein said compound is selected from the group consisting of organic and inorganic molecules, and wherein said compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay as outlined above for MN/CA IX using a saturated $C0_2$ solution.

Still further, this invention concerns novel compounds that are useful as CA IX-specific inhibitors in a variety of methods disclosed herein. Such novel compounds include pyridinium derivatives of heterocyclic sulfonamides with the general formula of:



wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, *n*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R2 is selected from the group consisting of hydrogen and methyl;

R3 is selected from the group consisting of hydrogen, methyl, *n*-nonyl and phenyl;

R4 is selected from the group consisting of hydrogen and methyl; and

R5 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl, *n*-nonyl and phenyl, except that

R1 cannot be methyl when R2 and R4 are hydrogen and R3 and R5 are methyl; and

R1 cannot be methyl when R2 and R4 are hydrogen, R3 is phenyl and R5 is methyl; and

R1 cannot be phenyl when R2 and R4 are hydrogen and R3 and R5 are phenyl. Preferred such pyridinium derivatives of heterocyclic sulfonamides include those wherein

R2 and R4 are hydrogen;

R3 is methyl; and

R1 and R5 are the same and selected from the group consisting of *iso*-propyl and *tert*-butyl, and those wherein

R2 and R4 are hydrogen;

R3 is phenyl; and

R1 and R5 are the same and selected from the group consisting of ethyl, iso-propyl, n-propyl, and n-butyl, and further preferably those wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, and *tert*-butyl;

R2 and R4 are hydrogen; and

R3 and R5 are phenyl. Still further preferred are those pyridinium derivatives of heterocyclic sulfonamides, wherein

R1 is iso-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen; or

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

R1, R2, R3 and R5 are methyl and R4 is hydrogen; or

R1 and R4 are methyl,R2 is hydrogen and R3 and R5 are *n*-nonyl.

In another therapeutic aspect of the invention, the CA IX-specific inhibitors can be conjugated to radioisotopes for administration. Also, the CA IX-specific inhibitors can be administred concurrently and/or sequentially with radiation and/or with a therapeutically effective amount in a physiologically acceptable formulation of one or more of the following compounds selected from the group

consisting of: conventional anticancer drugs, chemotherapeutic agents, different inhibitors of cancer-related pathways, bioreductive drugs, CA IX-specific antibodies and CA IX-specific antibody fragments that are biologically active. Preferably said CA IX-specific antibodies and/or CA IX-specific antibody fragments are humanized or fully human, and may be attached to a cytotoxic entity.

In another therapeutic aspect, this invention concerns methods of treating a mammal for a precancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprising administering to said mammal a therapeutically effective amount in a physiologically acceptable formulation of a vector conjugated to a potent CA IX-specific inhibitor, wherein said vector expresses a wild-type gene that is absent from or mutated in a CA IX expressing cell, that is precancerous or cancerous, and wherein the wild type gene product has an anticancer effect in said cell; or wherein said vector comprises a gene that expresses a cytotoxic protein. An exemplary wild-type gene would be the von Hippel-Lindau gene known to be directly involved in the constitutive expression of CA IX in renal cell carcinoma.

Preferably said vector comprises a MN/CA IX promoter or a MN/CA IX promoter fragment, wherein said promoter or promoter fragment comprises one or more hypoxia response elements (HREs), and wherein said promoter or promoter fragment is operably linked to said wild-type gene or to said gene that expresses a cytotoxic protein. Preferably the CA IX-specific inhibitor conjugated to the vector has a K_I as determined above in the $C0_2$ saturation assay to be less than about 50 nM, more preferably less than about 35 nM, still more preferably less than about 25 nM and still further more preferably less than about 10 nM. Preferably, said potent MN/CA IX inhibitor is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate.

Still in another aspect, this invention concerns methods that are diagnostic or diagnostic and prognostic for precancer or cancer. For example, such methods may comprise contacting a mammalian sample with a CA IX-specific inhibitor conjugated to a label or a visualizing means, and detecting or detecting and quantifying binding of said CA IX-specific inhibitor to cells in said sample by detecting or detecting and quantifying said label or said visualizing means on cells in

said sample, wherein said detection or said detection and quantitation at a level above that for a control sample is indicative of precancerous or cancerous cells that overexpress CA IX in said sample.

Such methods can be of particular diagnostic and prognostic importance by detecting or detecting and quantitating CA IX activated by hypoxic conditions. Hypoxia combined with CA IX overepression indicates that the mammal from whom the sample was taken is considered to have a poorer prognosis, and decisions on treatment for said mammal are made in view of the presence of said hypoxic conditions. MN/CA IX as a hypoxia marker is useful in general in making therapeutic decisions. For example, a cancer patient whose tumor is known to express MN/CA IX at an abnormally high level would not be a candidate for certain kinds of chemotherapy and radiotherapy, but would be a candidate for hypoxia-selective chemotherapy.

Brown, J.M. [*16*] points out at page 157 that "solid tumours are considerably less well oxygenated than normal tissues. This leads to resistance to radiotherapy and anticancer chemotherapy, as well as predisposing to increased tumour metastases." Brown explains how tumor hypoxia can be exploited in cancer treatment. One strategy to exploit tumor hypoxia for cancer treatment proposed by Brown [*16*] is to use drugs that are toxic only under hypoxic conditions. Exemplary and preferred drugs that could be used under that strategy include tirapazamine and AQ4N, a di-N-oxide analogue of mitozantrome.

A second mode of exploiting hypoxia proposed by Brown [*16*] is by gene therapy strategies developed to take advantage of the selective induction of HIF-1. Brown notes that a tumor-specific delivery system can be developed wherein a promoter that is highly responsive to HIF-1 would drive the expression of a conditionally lethal gene under hypoxic but not normoxic conditions. The MN/CA IX promoter is just such a promoter highly responsive to hypoxia, as well as MN/CA IX promoter fragments comprising one or more HREs. "Expression of an enzyme not normally found in the human body could, under the control of a hypoxia-responsive promoter [the MN/Ca IX promoter], convert a nontoxic pro-drug into a toxic drug in the tumour." [Brown [*16*], page 160.] Exemplary is the use of the bacterial cytosine

deaminase, which converts the nontoxic 5-fluorocytosine to the anticancer drug 5-fluorouracil (5FU) cited by Brown to Trinh et al. [*109*].

Ratcliffe et al., U.S. Patent Nos. 5,942,434 and 6,265,390 explain how anti-cancer drugs become activated under hypoxia [*119*], but that the use of a drug activation system, wherein the enzyme that activates the drug is significantly increased under hypoxia, results in much enhanced therapeutic effect.

This invention further concerns methods for imaging tumors and/or metastases that express CA IX in a patient comprising the administration of a CA IX-specific inhibitor linked to an imaging agent to said patient. A preferred imaging method would encompass scintigraphy.

The assays of this invention are both diagnostic and/or prognostic, i.e., diagnostic/prognostic. The term "diagnostic/ prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the presence of disease, determining the nature of a disease, distinguishing one disease from another, forecasting as to the probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods of this invention are useful, for example, for screening populations for the presence of neoplastic or pre-neoplastic disease, determining the risk of developing neoplastic disease, diagnosing the presence of neoplastic and/or pre-neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or determining the prognosis for the course of neoplastic disease.

The present invention is useful for treating and for screening the presence of a wide variety of preneoplastic/neoplastic diseases including carcinomas, such as, mammary, colorectal, urinary tract, ovarian, uterine, cervical, endometrial, squamous cell and adenosquamous carcinomas; head and neck cancers; mesodermal tumors, such as, neuroblastomas and retinoblastomas; sarcomas, such as osteosarcomas and Ewing's sarcoma; and melanomas. Of particular interest are gynecological cancers including ovarian, uterine, cervical, vaginal, vulval and endometrial cancers, particularly ovarian, uterine cervical and endometrial cancers.

Also of particular interest are cancers of the breast, of gastrointestinal tract, of the stomach including esophagus, of the colon, of the kidney, of the prostate, of the liver, of the urinary tract including bladder, of the lung, and of the head and neck. Gynecologic cancers of particular interest are carcinomas of the uterine cervix, endometrium and ovaries; more particularly such gynecologic cancers include cervical squamous cell carcinomas, adenosquamous carcinomas, adenocarcinomas as well as gynecologic precancerous conditions, such as metaplastic cervical tissues and condylomas.

The invention provides methods and compositions for evaluating the probability of the presence of malignant or pre-malignant cells, for example, in a group of cells freshly removed from a host. Such an assay can be used to detect tumors, quantitate their growth, and help in the diagnosis and prognosis of disease. The assays can also be used to detect the presence of cancer metastasis, as well as confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor reappearance.

The presence of MN antigen can be detected and/or quantitated using a number of well-defined diagnostic assays. Those in the art can adapt any of the conventional immunoassay formats to detect and/or quantitate MN antigen as herein disclosed. The immunoassays of this invention can be embodied in test kits which comprise the potent CA IX-specific inhibitors of this invention, appropriately labeled and/or linked to a visualizing means, as known in the art. Such test kits can be in solid phase formats, but are not limited thereto, and can also be in liquid phase format, and can be based on immunohistochemical assays, ELISAS, particle assays, radiometric or fluorometric assays either unamplified or amplified, using, for example, avidin/biotin technology, among other assay formats.

Exemplary CA IX-specific inhibitors of the invention are shown herein to treat transfected cells that constitutively express MN/CA IX compared to non-transfected cells with no MN/CA IX expression. The exemplary CA IX-specific inhibitors are shown to inhibit acidification of extracellular pH induced by MN/CA IX in cell cultures exposed to hypoxia.

Further, labeled exemplary CA IX-specific inhibitors, such as labeled sulfonamides, for example, conjugated to fluorescein isothiocyanate (FITC), are shown to bind to the surface of MN/CA IX transfected cells, and not to control cells, only in hypoxia but not in normoxia. Those experiments confirm that CA IX-specific inhibitors, such as the sulfonamide compounds described herein, can specifically target MN/CA IX under conditions characteristic of intratumoral microenvironments.

The CA IX-specific inhibitors of this invention can be used diagnostically and prognostically for precancer and cancer, and to determine the status of a patient, and therapeutically, individually or in different combinations with conventional therapeutic regimens to treat precancers and/or cancer. The CA IX-specific inhibitors may also be used in cancer research.

More particularly for treating precancer and/or cancer, the CA IX-specific inhibitors of this invention can be used to hinder cancer expansion and/or progression by blocking CA IX activity. The CA IX-specific inhibitors can be conjugated to radioisotopes for radiotherapy. The CA IX-specific inhibitors can be combined with CA IX-specific antibodies and a variety of conventional therapeutic drugs, different inhibitors of cancer-related pathways, bioreductive drugs, and/or radiotherapy, wherein different combinations of treatment regimens with the CA IX-specific inhibitors of this invention may increase overall treatment efficacy. Particularly, the CA IX-specific inhibitors of this invention may be combined with therapy using MN/CA IX-specific antibodies and/or CA IX-specific antibody fragments, preferably humanized CA IX-specific antibodies and/or biologically active fragments thereof, and more preferably fully human CA IX-specific antibodies and/or fully human CA IX-specific biologically active antibody fragments. Said CA IX-specific antibodies and biologically active CA IX-specific antibody fragments, preferably humanized and more preferably fully human, may be conjugated to a cytotoxic entity, for example, a cytotoxic protein, such as ricin A, among many other cytotoxic entities.

Still further, a CA IX-specific inhibitor of this invention could be coupled to a vector for targeted delivery to CA IX-specific expressing cells for gene therapy (for example, with the wild-type von Hippel-Lindau gene), or for effecting the expression of cytotoxic proteins, preferably wherein said vector comprises a MN/CA

IX promoter or MN/CA IX promoter fragment comprising the MN/CA IX hypoxia response element (HRE) or a HRE of another gene, and more preferably wherein the CA IX promoter or CA IX promotor fragment comprises more than one HRE, wherein said HRE or HREs is or are either of MN/CA IX, and/or of other genes and/or of genetically engineered HRE consensus sequences in a preferred context.

Particularly, the CA IX-specific inhibitors of this invention can be used diagnostically/prognostically to detect precancerous and/or cancerous cells by binding to CA IX, preferably to CA IX activated by hypoxic conditions, wherein said CA IX specific inhibitors are coupled to a label or to some visualizing means. Such detection, particularly of hypoxic conditions, and CA IX overexpression, can be helpful in determining effective treatment options, and in predicting treatment outcome and the prognosis of disease development. Further the CA IX-specific inhibitors when labeled or linked to an appropriate visualizing means can be used for imaging tumors and/or metastases that express CA IX.

The CA IX-specific inhibitors of this invention can also be used in basic and pre-clinical research. For example, the CA IX-specific inhibitors can be used to study the regulation of CA IX enzyme activity, to study the role of CA IX in tumor growth and metabolism, and to study the role of CA IX in response to treatment by drugs, radiation, inhibitors and other therapeutic regimens.

Further methods are disclosed for the preparation of positively-charged, membrane-impermeant heterocyclic sulfonamide CA inhibitors with high affinity for the membrane-bound carbonic anhydrase CA IX. Particularly preferred CA IX-specific inhibitors are pyridinium derivatives of such aromatic and heterocyclic sulfonamides. The general structure of the preferred pyridinium derivatives of sulfonamides can be described as a pyridinium portion attached to the “tail” of an aromatic or heterocyclic sulphonamide portion of the compound.

Further provided are screening assays for compounds that are useful for inhibiting the growth of a vertebrate, preferably mammalian, more preferably human, preneoplastic or neoplastic cell that abnormally expresses MN protein. Said screening assays comprise tests for the inhibition of the enzymatic activity of MN by said compounds. Additional assays provided herein test said compounds for their cell membrane impermeance.

Aspects of the instant invention disclosed herein are described in more detail below.

References

The following references are cited throughout the application by numbers in italics keyed to the list below:

1. Abbate et al., J. Med. Chem., 45: 3583-3587 (2002).
2. Abbate et al., J. Enz. Inhib. Med. Chem., 18: 303-308 (2003a).
3. Abbate et al., Bioorg. Med. Chem. Lett., 13: In Press (2003b).
4. Abdine et al., J. Assoc. Off. Anal. Chem., 61: 695-701 (1978).
5. Aldred et al., Biochemistry, 30: 569-575 (1991).
6. Balaban et al., "Pyrylium Salts: Syntheses, Reactions and Physical Properties," In Advances in Hetercyclic Chemistry, Katritzky, A.R., Ed., Academic Press, New York, pp. 8-360 (1982).
7. Barlow et al., Nucl. Acids Res., 15: 2386 (1987).
8. Bartosova et al., J. Pathol., 197: 314-321 (2002).
9. Bayer, A., Ber. Dtsch. Chem. Ges., 43: 2337-2349 (1910).
10. Bayer and Piccard, Liebigs Ann. Chem., 384: 208-223 (1911).
11. Beasley et al., Cancer Res., 61: 5262-5267 (2001).
12. Behravan et al., Eur. J. Biochem, 190: 351-357 (1990).
13. Borras et al., Bioorg. Med. Chem., 7: 2397-2406 (1999).
14. Briganti et al., Biochemistry, 36: 10384-10392 (1997).
15. Briganti et al., Inorg. Chim. Acta.: 275-276, 295-300 (1998).
16. Brown, J.M., "Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies," Molecular Medicine Today, 6: 157-162 (April 2000).
17. Casini et al., J. Med. Chem., 43: 4884-4892 (2000).
18. Casini et al., Curr. Cancer Drug Targets, 2: 55-75 (2002).
19. a) Casini et al., Bioorg. Med. Chem. Lett, 13: 841-845 (2003)
    b) Casini et al., Biorg. Med. Chem. Lett., 13: 2763-2769 (2003).

20. Chegwidden et al., "The Roles of carbonic anhydrase isozymes in cancer," Gene Families: Studies of DNA, RNA, Enzymes and Proteins, Xue et al., Eds., World Scientific, Singapore, pp. 157-169 (2001).

21. Chia et al., J. Clin. Oncol., 19: 3660-3668 (2001).

22. Chirica et al., Biochim. Biophys. Acta, 1544: 55-63 (2001).

23. Clare and Supuran, Eur. J. Med., Chem.: 32: 311-319 (1997).

24. Clare and Supuran, Eur. J. Med. Chem., 34: 463-474 (1999).

25. Cuthbert et al., J. Physiol., 551 (Pt.1) 79-92, (2003).

26. Dinculescu and Balaban, Rev. Roum. Chem., 25: 1505-1528 (1980).

27. Dodgson et al., The Carbonic Anhydrases, Plenum Press, New York-London, pp. 398 (1991).

28. Doege et al., J. Biol. Chem., 266: 894-902 (1991).

29. Elleby et al., Eur. J. Biochem., 268: 1613-1619 (2001).

30. Ferraroni et al., Biochemistry, 41: 6237-6244 (2002a).

31. Ferraroni et al., Inorg. Chim. Acta, 339: 135-144 (2002b).

32. Fleming et al., J. Clin. Invest., 96: 2907-2913 (1995).

33. Franchi et al., J. Enz. Inhib. Med. Chem., 18: 333-338 (2003).

34. Geers and Gros, Physiol. Rev. 80:681-715 (2000).

35. Giatromanolaki et al., Cancer Res., 61: 7992-7998 (2001).

36. Gomaa, Z.S., Biomed. Chromatogr., 7: 134-135 (1993).

37. Gruneberg et al., Angew. Chem. Int. Ed., 40: 389-393 (2001).

38. Hakansson et al., J. Mol. Biol., 227: 1192-1204 (1992).

39. Heming et al., J. Appl. Physiol., 61: 1849-1856 (1986).

40. Hewett-Emmett, D., "Evolution and distribution of the carbonic anhydrase gene families," In The Carbonic Anhydrases – New Horizons, Chegwidden et al., Eds., Birkhauser Verlag: Basel, Switzerland, pp. 29-78 (2000).

41. Höckel and Vaupel, J. Natl. Cancer Inst., 93: 266-276 (2001).

42. Ilies et al., Bioorg. Med. Chem., 8: 2145-2155 (2000).

43. Ilies et al., J. Med. Chem., 46: 2187-2196 (2003).

44. Khalifah, R.G., J. Biol. Chem., 246: 2561-2573 (1971).

45. Khalifah et al., Biochemistry, 16: 2241-2247 (1977).

46. Kim et al., J. Am. Chem. Soc., 122: 12125-12134 (2000).

47. Kim et al., J. Am. Chem. Soc., 123: 9620-9627 (2001).
48. Koukourakis et al., Clin. Cancer Res., 7: 3399-3403 (2001).
49. Krungkrai et al., Int. J. Parasitol., 31: 661-668 (2001).
50. Liao et al., Am. J. Pathol., 145: 598-609 (1994).
51. Liao et al., Cancer Res., 57: 2827-2831 (1997).
52. Lieskovska et al., Neoplasma, 46: 17-24 (1999).
53. Lindskog and Coleman, Proc. Natl. Acad. Sci. (USA) 70: 2505-2508 (1964).
54. Lindskog et al., "Structure-function relations in human carbonic anhydrase II as studied by site-directed mutagenesis," in Carbonic anhydrase – From biochemistry and genetics to physiology and clinical medicine, Botre et al., Eds., VCH, Weinheim, pp. 1-13 (1991)].
55. Lloyd et al., Genes. Dev., 1: 594-602 (1987).
56. Loncaster et al., Cancer Res., 61: 6394-6399 (2001).
57. Maren, T.H., Physiol. Rev., 47: 595-781 (1967).
58. Maren, T.H., "Benzolamide – a renal carbonic anhydrase inhibitor," In Orphan Drugs, Karch, T.E., Ed., M. Dekker, New York, pp. 89-115 (1982).
59. Maren et al., Mol. Pharmacol., 44: 901-906 (1993).
60. Maren et al., J. Pharmacol. Exp. Ther., 280: 98-104 (1997).
61. Mendelsohn and Lippman, "Growth Factors," pp. 114-133, IN: DeVita et al. (eds.), Cancer: Principles and Practice of Oncology (4^{th} Ed.; Lippincott; Philadelphia, 1993).
62. Mincione et al., Eur. J. Pharm. Sci., 9: 185-199 (1999).
63. Montgomery et al., Nucl. Acids. Res., 15: 4687 (1987).
64. Mori et al., Gastroenterol., 105: 820-826 (1993).
65. Okuyama et al., PNAS (USA) 89: 1315-1319 (1992).
66. Opavsky et al., Genomics, 33: 480-487 (1996).
67. Owa and Nagasu, Exp. Opin. Ther. Patents, 10: 1725-1740 (2000).
68. Owa et al., J. Med. Chem., 42: 3789-3799 (1999).
69. Parkkila et al., Gut, 35: 646-650 (1994).
70. Parkkilla et al., Histochem. J., 27: 133-138 (1995).
71. Parkkila et al., Hepatology, 24: 104 (1996).
72. Pastorek et al., Oncogene, 9: 2788-2888 (1994).

73. Pastorekova et al., Virology, 187: 620-626 (1992).
74. Pastorekova et al., Gastroenterology, 112: 398-408 (1997).
75. Pearson and Lipman, PNAS (USA), 85: 2444 (1988).
76. Pocker and Stone, Biochemistry, 6: 668-678 (1967).
77. Scozzafava and Supuran, Bioorg. Med. Chem. Lett., 10: 117-1120 (2000).
78. Scozzafava et al., Curr. Med. Chem., 10: 925-953 (2003).
79. Scozzafava et al., J. Med. Chem., 42: 2641-2650 (1999).
80. Scozzafava et al., J. Med. Chem., 42: 3690-3700 1999)
81. Scozzafava et al., J. Med. Chem., 43: 292-300 (2000).
82. Sly and Hu, Annu. Rev. Biochem., 64: 375-401 (1995).
83. Smith and Ferry, FEMS Microbiol. Rev., 24: 335-366 (2000).
84. Steiner et al., Eur. J. Biochem., 59: 253-259 (1975).
85. Sterling et al., Am. J. Physiol.-Cell Physiol., 283: C1522-C1529 (2002).
86. Stubbs et al., Mol. Med. Today, 6: 15-19 (2000).
87. Supuran, C.T., Opin. Investig. Drugs, 12: 283-287 (2003).
88. Supuran and Clare, Eur J. Med. Chem., 30: 687-696 (1995).
89. Supuran and Clare, Eur J. Med. Chem., 33: 489-500 (1998).
90. Supuran and Clare, Eur. J. Med. Chem., 34: 41-50 (1999).
91. Supuran and Scozzafava, Enzyme Inhib., 15: 597-610 (2000a).
92. Supuran and Scozzafava, Eur. J. Med. Chem., 35: 867-874 (2000b).
93. Supuran and Scozzafava, Exp. Opin. Ther. Patents,10: 575-600 (2000c).
94. Supuran and Scozzafava, Curr. Med. Chem.- Imm., Endoc. Metab. Agents, 1: 61-97 (2001).
95. Supuran and Scozzafava, Exp. Opin. Ther. Patents, 12: 217-242 (2002).
96. Supuran et al., Eur. J. Med. Chem., 33: 577-594 (1998a).
97. Supuran et al., Eur. J. Med. Chem., 33: 739-752 (1998b).
98. Supuran et al., J. Enz. Inhib., 15: 381-401 (2000a).
99. Supuran et al., J. Med. Chem., 35: 309-321 (2000b).
100. Supuran et al., Bioorg. Med. Chem., 9: 703-714 (2001a).
101. Supuran et al., Curr. Med. Chem.- Imm., Endoc. Metab. Agents, 1: 61-97 (2001b)
102. Supuran et al., Med. Res. Rev., 23: 146-189 (2003).

103. Svastova et al., Experimental Cell Research, 290: 332-345, (2003).

104. Symington, J. Biol. Chem., 267: 25744 (1992).

105. Tashian, R. E., Adv. in Genetics, 30: 321-356 (1992)..

106. Teicher et al., Anticancer Research, 13: 1549-1556 (1993).

107. Tinker et al., J. Pharmacol. Exp. Ther., 218: 600-607 (1981).

108. Toma and Balaban, Tetrahedron, Suppl. 7: 27-34 (1966).

109. Trinh et al., Cancer Res., 55: 4808-4812 (1995).

110. Turner, et al., Hum. Pathol., 28: 740-744 (1997).

111. Turner et al., Br. J. Cancer, 86: 1276-1282 (2002).

112. Uemura et al. [J. Urology, 1571 (4 Supp.): 377 (Abstract 1475) (April 16, 1997)]

113. Vermylen et al., Eur. Respir. J., 14: 806-811 (1999);

114. Vullo et al., Bioorg. Med. Chem. Lett., 13: 1005-1009 (2003a).

115. Vullo et al. J. Enz. Inhib. Med. Chem., 18: 403-406 (2003b).

116. Wingo et al., Biochem. Biophys. Res. Comm., 288: 666-669 (2001).

117. Winum et al., J. Med. Chem., 46: 2197-2204 (2003).

118. Wistrand and Lindqvist, "Design of carbonic anhydrase inhibitors and the relationship between the pharmacodymanics and pharmacokinetics of acetazolamide," In Carbonic Anhydrase – FromBiochemistry and Genetics to Physiology and Clinical Medicine, Botrè et al., Eds., VCH, Weinheim, pp. 352-378 (1991).

119. Workman and Stafford, Cancer and Metastasis Reviews, 12: 73-82 (1993)

120. Wu et al., J. Membr. Biol. 162: 31-38 (1998).

121. Wykoff et al., Cancer Research, 60: 7075-7083 (2000).

122. Wykoff et al., Am. J. Pathol., 158: 1011-1019 (2001).

123. Zavada et al., Int. J. Cancer, 54: 268-274 (1993).

Abbreviations

The following abbreviations are used herein:

aa	-	amino acid
AAZ	-	acetazolamide
ATCC	-	American Type Culture Collection

bp	-	base pairs
BRL	-	Bethesda Research Laboratories
BRZ	-	brinzolamide
BSA	-	bovine serum albumin
CA	-	carbonic anhydrase
CAI	-	carbonic anhydrase inhibitor
CAM	-	cell adhesion molecule
CARP	-	carbonic anhydrase related protein
Ci	-	curie
cm	-	centimeter
CNS	-	central nervous system
cpm	-	counts per minute
C-terminus	-	carboxyl-terminus
°C	-	degrees centigrade
DCP	-	dichlorophenamide
DEAE	-	diethylaminoethyl
DMEM	-	Dulbecco modified Eagle medium
ds	-	double-stranded
DZA	-	dorzolamide
EDTA	-	ethylenediaminetetraacetate
EZA	-	ethoxzolamide
F	-	fibroblasts
FCS	-	fetal calf serum
FITC	-	fluorescein isothiocyanate
H	-	HeLa cells
IC	-	intracellular
kb	-	kilobase
kbp	-	kilobase pairs
kd or kDa	-	kilodaltons
K_I	-	inhibition constant
KS	-	keratan sulphate
LTR	-	long terminal repeat

M	-	molar
mA	-	milliampere
MAb	-	monoclonal antibody
ME	-	mercaptoethanol
MEM	-	minimal essential medium
min.	-	minute(s)
mg	-	milligram
ml	-	milliliter
mM	-	millimolar
MMC	-	mitomycin C
mmol	-	millimole
MZA	-	methazolamide
N	-	normal concentration
NEG	-	negative
ng	-	nanogram
nm	-	nanometer
nM	-	nanomolar
nt	-	nucleotide
N-terminus	-	amino-terminus
ODN	-	oligodeoxynucleotide
ORF	-	open reading frame
PA	-	Protein A
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PG	-	proteoglycan
pI	-	isoelectric point
PMA	-	phorbol 12-myristate 13-acetate
POS	-	positive
Py	-	pyrimidine
QAS	-	quaternary ammonian sulfonilamide
QSAR	-	quantitative structure-activity relationship(s)
RACE	-	rapid amplification of cDNA ends

RCC	-	renal cell carcinoma
RIA	-	radioimmunoassay
RIP	-	radioimmunoprecipitation
RIPA	-	radioimmunoprecipitation assay
RNP	-	RNase protection assay
RT-PCT	-	reverse transcription polymerase chain reaction
SAC	-	<u>Staphylococcus aureus</u> cells
SAR	-	structure-activity relationship
sc	-	subcutaneous
SDS	-	sodium dodecyl sulfate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SINE	-	short interspersed repeated sequence
SP	-	signal peptide
SP-RIA	-	solid-phase radioimmunoassay
TBE	-	Tris-borate/EDTA electrophoresis buffer
TC	-	tissue culture
TCA	-	trichloroacetic acid
TC media	-	tissue culture media
TC	-	tissue culture
tk	-	thymidine kinase
TM	-	transmembrane
Tris	-	tris (hydroxymethyl) aminomethane
μCi	-	microcurie
μg	-	microgram
μl	-	microliter
μM	-	micromolar

<u>Cell Lines</u>

BL21 (DE3) -- *Escherichia coli* strain described by Lindskog's group (for CA I, II expression)[Lindskog et al., "Structure-function relations in human carbonic anhydrase II as studied by site-directed

mutagenesis," in Carbonic anhydrase – From biochemistry and genetics to physiology and clinical medicine, Botre et al., Eds., VCH, Weinheim, pp. 1-13 (1991)]

BL21-GOLD -- (DE3)	*Escherichia coli* strain (from Stratagene) used for CA IX expression)

Nucleotide and Amino Acid Sequence Symbols

The following symbols are used to represent nucleotides herein:

Base Symbol	Meaning
A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil
I	inosine
M	A or C
R	A or G
W	A or T/U
S	C or G
Y	C or T/U
K	G or T/U
V	A or C or G
H	A or C or T/U
D	A or G or T/U
B	C or G or T/U
N/X	A or C or G or T/U

There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-

letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figure 1 as follows:

Amino acid name	3 Ltr. Abbrev.	1 Ltr. Abbrev.
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown or other		X

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-C provides the nucleotide sequence for MN/CA IX full-length cDNA [SEQ ID NO: 1]. Figure 1 A-C also sets forth the predicted amino acid sequence [SEQ ID NO: 2] encoded by the cDNA.

Figure 2A-F provides a 10,898 bp complete genomic sequence of MN/CA9 [SEQ ID NO: 3]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are in general shown in capital letters, but exon 1 is considered to begin at position 3507 as determined by RNase protection assay.

Figure 3 provides an exon-intron map of the human MN/CA9 gene. The positions and sizes of the exons (numbered, cross-hatched boxes), Alu repeat elements (open boxes) and an LTR-related sequence (first unnumbered stippled box) are adjusted to the indicated scale. The exons corresponding to individual MN/CA IX protein domains are enclosed in dashed frames designated PG (proteoglycan-like domain), CA (carbonic anhydrase domain), TM (transmembrane anchor) and IC (intracytoplasmic tail). Below the map, the alignment of amino acid sequences illustrates the extent of homology between the MN/CA IX protein PG region (aa 53-111) [SEQ ID NO: 4] and the human aggrecan (aa 781-839) [SEQ ID NO: 5].

Figure 4 A-B shows the chemical structures of the 26 different sulfonamide compounds tested in Example 1.

Figure 5 shows the scheme for the general synthesis of compounds **71 – 91** of Example 3 (**Scheme 1**).

Figure 6 shows the scheme for the reaction between a pyrylium salt and an amine (**Scheme 2**), as described in Example 3.

DETAILED DESCRIPTION

The novel methods of the present invention comprise inhibiting the growth of tumor cells which overexpress MN protein with compounds that inhibit the enzymatic activity of MN protein. Said compounds are organic or inorganic, preferably organic, more preferably sulfonamides. Still more preferably, said compounds are pyridinium derivatives of aromatic or heterocyclic sulfonamides. These preferred pyridinium derivatives of sulfonamides are likely to have fewer side effects than other compounds in three respects: they are small molecules, they are membrane-impermeant, and they are specific potent inhibitors of the enzymatic activity of the tumor-associated MN/CA IX protein.

The use of oncoproteins as targets for developing new cancer therapeutics is considered conventional by those of skill in the art. [<u>See</u>, e.g.,

Mendelsohn and Lippman [*61*]. However, the application of such approaches to MN is new. In comparison to other tumor-related molecules (e.g. growth factors and their receptors), MN has the unique property of being differentially expressed in preneoplastic/neoplastic and normal tissues, which are separated by an anatomic barrier.

The pyridinium derivatives of sulfonamides of the present invention can be formed, for example, by creating bonds between pyrylium salts and aromatic or heterocyclic sulfonamide reagents, as described below. The aromatic or heterocyclic sulfonamide portion of a pyridinium salt of a sulfonamide compound can be called the "head," and the pyridinium portion can be called the "tail."

It can be appreciated by those of skill in the art that various other types of linkages can couple the pyridinium portion with the sulfonamide portion. It can further be appreciated that alternate methods, in addition to those disclosed herein, can be used to make the pyridinium derivatives of the present invention.

As used herein, "cancerous" and "neoplastic" have equivalent meanings, and "precancerous" and "preneoplastic" have equivalent meanings.

As used herein, the term "aromatic" when applied to sulphonamide structures means "comprising an aromatic ring, without an additional heterocyclic ring." The term "heterocyclic" when applied to sulphonamide structures means "comprising a heterocyclic ring, with or without an additional aromatic ring."

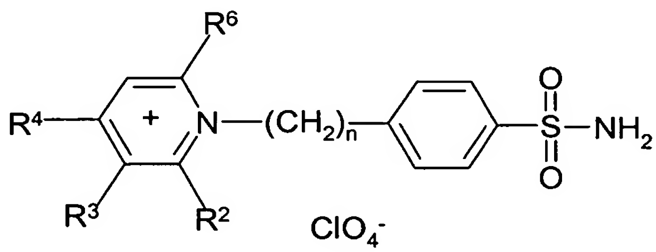
As used herein, the term "alkyl", alone or in combination, refers to a straight-chain or branched-chain alkyl radical containing from 1 to 12, preferably from 1 to 6 and more preferably from 1 to 4, carbon atoms. Examples of such radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, decyl and the like.

The term "aryl", alone or in combination, means a phenyl or naphthyl radical which optionally carries one or more substituents selected from alkyl, alkoxy, halogen, hydroxy, amino, nitro, cyano, haloalkyl, carboxy, alkoxycarbonyl, cycloalkyl, heterocycloalkyl, amido, mono and dialkyl substituted amino, mono and dialkyl substituted amido and the like, such as phenyl, p-tolyl, 4-methoxyphenyl, 4-(tert-butoxy)phenyl, 3-methyl-4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 3-nitrophenyl, 3-aminophenyl, 3-acetamidophenyl, 4-acetamidophenyl, 2-methyl-3-

acetamidophenyl, 2-methyl-3-aminophenyl, 3-methyl-4-aminophenyl, 2-amino-3-methylphenyl, 2,4-dimethyl-3-aminophenyl, 4-hydroxyphenyl, 3-methyl-4-hydroxyphenyl, 1-naphthyl, 2-naphthyl, 3-amino-1-naphthyl, 2-methyl-3-amino-1-naphthyl, 6-amino-2-naphthyl, 4,6-dimethoxy-2-naphthyl and the like.

Preferred sulfonamides of the present invention are aromatic and heterocyclic sulfonamides. The structures of representative sulfonamides of this group, designated **1-26,** are shown in Figure 4.

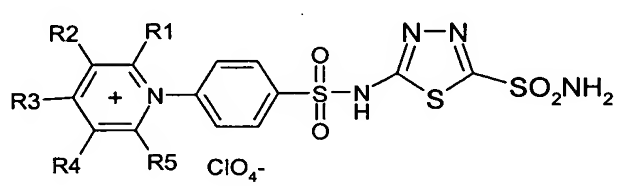
More preferred sulfonamides of the present invention are pyridinium derivatives of aromatic sulfonamides and have the general formula (**A**) below,



A

wherein n is 0, 1, or 2; and R2, R3, R4 and R6 are each independently selected from the group consisting of hydrogen, alkyls and aryls. The structures of representative sulfonamides of this group, designated **27** through **70**, are shown as derivatives of the general structure **(A),** in Table 2.

Alternatively, more preferred sulfonamides of the present invention are pyridinium derivatives of heterocyclic sulfonamides and have the general formula (**B**) below, wherein said pyridinium derivative of a heterocyclic sulfonamide has the general formula of:



B

wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, akyls and aryls. The structures of representative sulfonamides of this group, designated **71** through **91**, are shown as derivatives of the general structure (**B**), in Table 3.

Representative sulfonamide derivatives of the group of compounds represented by the general formulas (**A**) and (**B**) have CA IX inhibitory activity, and are potentially useful therapeutically as anticancer agents in treating MN-associated tumors.

Further, biologic activity of the identified sulfonamides will be tested in vitro by inhibition of the carbonic anhydrase enzymatic activity of the MN protein, by effects on cell morphology and growth characteristics of MN-related tumor cells (HeLa) and of control cells [*104*]. In vivo screening will be carried out in nude mice that have been injected with HeLa cells.

<u>Representative Sulfonamide Inhibitors of CA IX</u>

The sulfonamides investigated in Example 1 for the inhibition of the tumor-associated isozyme CA IX, of types **1-26** are shown in Figure 4A-B. Compounds **1-6**, **11-12**, **20** and **26** are commercially available, whereas **7-10** [*43*], **13-19** [*24, 90, 97*] and **21-25** [*79*] were prepared as reported earlier. The six clinically used compounds were also assayed. For Example 2 compounds (pyridinium derivatives of aromatic sulfonamides), reaction of sulfanilamide, homosulfanilamide or 4-(2-aminoethyl)-benzenesulfonamide with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **27-70** investigated here, by the general Bayer - Piccard synthesis [*9,10, 97*].

As described in Example 3, a series of positively-charged sulfonamides, designated here as compounds **71-91**, were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrilium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring (described below). Three of these compounds (**71, 75,** and **87**) have been described elsewhere [*25, 85*]; all other compounds of this series are new.

Heterocyclic Sulfonamide Inhibitors of CA IX:
Synthesis of Pyridinium Derivatives of Aminobenzolamide

Chemistry: Reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) [*97*] with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **71-91** investigated here, by the general synthesis of such derivatives with nucleophiles (Scheme 1 as shown in Figure 5) [*6, 26, 108*].

Preparation of compounds: A large number of positively-charged sulfonamides, prepared by reaction of amino-sulfonamides with pyrylium salts [*23, 88, 89*] were recently reported by this group, and generally tested as inhibitors of the "classical" isozymes CA I, II and IV [*81, 96, 97, 98*]. Based on QSAR studies on several series of CA inhibitors, including some positively-charged derivatives [*23, 88, 89*], it emerged that the enhancement of CA inhibitory activity is correlated with increased positive charges on the heterocyclic/aromatic ring incorporated in such molecules, as well as with "long" inhibitor molecules *per se* (i.e., molecules extending on the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor) [*23, 88, 89*]. It appeared thus of interest to try to explore this result, designing positively-charged, long sulfonamide CAIs. Thus, we thought of attaching substituted-pyridinium moieties to an already potent and long-molecule CAI suitable for reaction with pyrylium salts, i.e., aminobenzolamide [*97*]. Indeed, this compound acts as a very potent CAI against isozymes I, II and IV (with inhibition constants in the low nanomolar range – see later in the text). The substitution pattern of the pyridinium ring was previously shown [*81, 96, 97, 98*] to be critical for the biological activity of this type of sulfonamide CAIs. Thus, a large series of of 2,4,6-trialkylpyridinium-; 2,6-dialkyl-4-phenylpyridinium-; 2-alkyl-4,6-diphenylpyridinium-; 2,4,6-triphenylpyridinium-, together with various 2,6-disubstituted-pyridinium and 2,3,5,6-tetrasubstituted-pyridinium aminobenzolamide derivatives have been prepared by the reaction described in Scheme 1 (Shown in Figure 5).

Although apparently simple, the reaction between a pyrylium salt and an amine, leading to pyridinium salts, is in reality a complicated process (Scheme 2, shown in Figure 6), as established by detailed spectroscopic and kinetic data from

Balaban's and Katritzky's groups [*6, 26, 108*]. Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the formation of intermediates of type **IV** (depicted in Figure 6), which by deprotonation in the presence of bases lead to the 2-amino-tetradehydropyran derivatives **V**. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough (this being the reason why in many cases one works at molar ratios pyrylium : amine of 1:2 when pyridinium salts are prepared by this method), or by external catalysts added to the reaction mixture, such as triethylamine [*6, 26, 108*]. The derivatives **V** are generally unstable, being tautomers with the ketodieneamines **VI** which are the key intermediates for the conversion of pyryliums into pyridiniums [*6, 26, 108*]. In acidic media, in the rate-determining step of the whole process, ketodieneamines **VI** may be converted to the corresponding pyridinium salts **VII**, although other products, such as vinylogous amides with diverse structures have also been isolated in such reactions [*6, 26, 108*]. A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl, cases in which a concurrent cyclisation with formation of the anilines **VIII** in addition to the pyridinium salts **VII**, may take place too [*6, 26, 108*]. These concurrent reactions mentioned above are generally important when the amine to be converted into the pyridinium salt possesses weak nucleophilicity or basicity. This happens to be the case of aminobenzolamide. In fact, reaction of aminobenzolamide with several pyrylium salts, performed in a variety of conditions (different solvents, such as low molecular weight alcohols (MeOH, EtOH, *i*-PrOH); DMF; methylene chloride; acetonitrile; diverse molar ratios of the reagents; temperatures from 25 to 150 °C; reaction times between 15 min and 48 hours, etc) led only to the isolation of the unreacted raw materials. The only conditions which led to the formation of the pyridinium salts **III** (depicted in Figure 5) were the following: anhydrous methanol in the presence of acetic anhydride as solvent and triethylamine as catalysts for the deprotonation of the intermediates **IV**. Acetic anhydride had the role of reacting with the water formed in the condensation reaction. This water may in fact act as a competitive nucleophile with aminobenzolamide when reacting with the pyrylium cation, and as a consequence the yields in pyridinium salts would dramatically be decreased. After the rapid formation of the ketodieneamine, catalyzed by

triethylamine (and in the presence of the acetic anhydride as water scavenging agent), the cyclisation to the pyridinium ring (the rate-determining step) has been achieved by refluxation in the presence of acetic acid (2-5 hours). Still the yields were not always good, especially for the 2-methyl-containing derivatives.

Preparation of MN Proteins and/or Polypeptides

The terms “MN/CA IX” and “MN/CA9” are herein considered to be synonyms for MN. Also, the G250 antigen is considered to refer to MN protein/polypeptide [*112*].

Zavada et al., WO 93/18152 and/or WO 95/34650 disclose the MN cDNA sequence shown herein in Figure 1A-1C [SEQ ID NO: 1], the MN amino acid sequence [SEQ ID NO: 2] also shown in Figure 1A-1C, and the MN genomic sequence [SEQ ID NO: 3] shown herein in Figure 2A-2F. The MN gene is organized into 11 exons and 10 introns.

The first thirty seven amino acids of the MN protein shown in Figure 1A-1C is the putative MN signal peptide [SEQ ID NO: 6]. The MN protein has an extracellular domain [amino acids (aa) 38-414 of Figure 1A-1C [SEQ ID NO: 7], a transmembrane domain [aa 415-434; SEQ ID NO: 8] and an intracellular domain [aa 435-459; SEQ ID NO: 9]. The extracellular domain contains the proteoglycan-like domain [aa 53-111: SEQ ID NO: 4] and the carbonic anhydrase (CA) domain [aa 135-391; SEQ ID NO: 5].

The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according to this invention has the deduced amino acid sequence shown in Figure 1. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figure 1. For example, such substantially homologous MN proteins/polypeptides are those that are reactive with the MN-specific antibodies, preferably the Mab M75 or its equivalent. The VU-M75 hybridoma that secretes the M75 Mab was deposited at the ATCC under HB 11128 on September 17, 1992.

A "polypeptide" or “peptide” is a chain of amino acids covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids. The term polypeptide encompasses the terms peptide and oligopeptide.

It can be appreciated that a protein or polypeptide produced by a neoplastic cell in vivo could be altered in sequence from that produced by a tumor cell in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid substitutions, extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN proteins that are significantly truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass MN proteins and/or polypeptides.

It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and result in proteins or polypeptides which are within the scope of this invention, as well as, MN muteins.

The MN proteins and polypeptides of this invention can be prepared in a variety of ways according to this invention, for example, recombinantly, synthetically or otherwise biologically, that is, by cleaving longer proteins and polypeptides enzymatically and/or chemically. A preferred method to prepare MN proteins is by a recombinant means. Particularly preferred methods of recombinantly producing MN proteins are described below. A representative method to prepare the MN proteins shown in Figure 1 or fragments thereof would be to insert the full-length or an appropriate fragment of MN cDNA into an appropriate expression vector as exemplified in the Materials and Methods section.

MN Gene

Figure 1A-C provides the nucleotide sequence for a full-length MN cDNA clone [SEQ ID NO: 1] isolated as described in Zavada et al., WO 95/34650. Figure 2A-F provides a complete MN genomic sequence [SEQ ID NO: 3].

The ORF of the MN cDNA shown in Figure 1 has the coding capacity for a 459 amino acid protein with a calculated molecular weight of 49.7 kd. The overall amino acid composition of the MN/CA IX protein is rather acidic, and predicted to have a pI of 4.3. Analysis of native MN/CA IX protein from CGL3 cells by two-dimensional electrophoresis followed by immunoblotting has shown that in agreement with computer prediction, the MN/CA IX is an acidic protein existing in several isoelectric forms with pIs ranging from 4.7 to 6.3.

The CA domain is essential for induction of anchorage independence, whereas the TM anchor and IC tail are dispensable for that biological effect. The MN protein is also capable of causing plasma membrane ruffling in the transfected cells and appears to participate in their attachment to the solid support. The data evince the involvement of MN in the regulation of cell proliferation, adhesion and intercellular communication.

Enzymatic Screening Assays

Assays are provided herein for the screening of compounds for inhibition of the enzymatic activity of the MN protein. Such assays comprise the incubation of said compound with said MN protein and with a substrate selected from the group consisting of saturated CO_2 and 4-nitrophenylacetate, preferably saturated CO_2, and determination of the inhibition constant K_I of said compound, wherein said enzymatic activity of the MN protein is measured by the pH change of an indicator by stopped flow spectrophotometer.

Screening of representative heterocyclic and aromatic sulfonamides for inhibition of MN protein: From Example 1, it was found that the inhibition profile of isozyme CA IX is very different from that of the classical isozymes CA I and II (cytosolic) as well as CA IV (membrane-bound). The following particular features may be noted: (i) all the 32 sulfonamides investigated in Example 1 act as CA IX inhibitors, with inhibition constants in the range of 14-285 nM (the corresponding

affinities for the other three isozymes vary in a much wider range, as seen from data of Table 1). Based on these data, it can be noted that CA IX is a sulfonamide avid CA, similarly to CA II, the isozyme considered up to now to be responsible for the majority of pharmacological effect of sulfonamides [*22, 29, 83,93, 94, 95, 102*]. Still, many other differences are observed between CA IX and other isozymes for which inhibitors were developed for clinical use; (ii) for CA I, II and IV, generally, aromatic sulfonamides behave as weaker inhibitors as compared to heterocyclic derivatives (compare **1-6**, or **DCP**), as aromatic compounds, with **15**, **21**, **AAZ**, **MZA**, **EZA**, **DZA** or **BRZ** among others (as heterocyclic sulfonamides). In the case of CA IX, such a fine distinction is rather difficult to be made, since both aromatic (such as **1**, **6**, **11**, **12**, **17**, **18**, **22-26**) derivatives, as well as heterocyclic compounds (such as **14**, **15**, **21**, and the clinically used sulfonamides – except dichlorophenamide) possess rather similar inhibition constants, in the range of 14-50 nM; (iii) orthanilamide derivatives (such as **1**, **17** and **22**) behave as very potent CA IX inhibitors (K_I-s in the range of 20-33 nM), although they are weak or medium-weak inhibitors of CA I, II and IV; (iv) 1,3-benzene-disulfonamide derivatives (such as **11**, **12** and **DCP**) are again strong CA IX inhibitors, with K_I-s in the range of 24-50 nM, although their CA II, I and IV inhibition profile is not particularly strong; (v) metanilamide **2**, sulfanilamide **3**, and 4-hydrazino-benzenesulfonamide **4** show CA IX inhibition data quite similar with those against CA II, whereas homosulfanilamide **5** and 4-aminoethyl-benzensulfonamide **6** act as better CA IX inhibitors as compared to CA II inhibition; (vi) the halogenosulfanilamides **7-10** are much weaker inhibitors of CA IX than of CA II, a finding difficult to interpret at this moment; (vii) the strongest CA II inhibitor among the investigated compounds, 4-aminobenzolamide **15** (K_I of 2 nM) is not the strongest CA IX inhibitor (K_I of 38 nM). Instead, the best CA IX inhibitor detected so far is the ethoxzolamide phenol **21** (K_I of 14 nM). It is interesting to note that **21** and **EZA** have the same affinity for CA II, whereas their affinity for CA IX is rather different, with the phenol more active than the ethoxy-derivative; (viii) among the clinically used compounds, the best inhibitor is acetazolamide, followed by methazolamide, ethoxzolamide and brinzolamide. The most ineffective (but appreciably inhibiting the isozyme IX) are dichlorophenamide and dorzolamide; (ix) sulfonamides **20** and **22-26** behave as very good CA IX inhibitors, with K_I-s in the

range of 16-32 nM, being slightly more effective than the clinically used CAIs mentioned above, and among the best CA IX inhibitors detected so far. It is thus envisageable that such compounds may be used as lead molecules for obtaining more potent and eventually specific CA IX inhibitors, with applications as antitumor agents.

Screening of representative pyridinium derivatives of aromatic sulfonamides for inhibition of MN protein: From Example 2, wherein membrane-impermeant pyridinium derivatives of sulfonamides were tested for their ability to inhibit the enzymatic activity of CA IX, the following conclusions were drawn from data of Table 2: (i) for a given substitution pattern of the pyridinium ring, the 4-aminoethyl-benzenesulfonamide derivatives **55-70** were more active than the corresponding homosulfanilamide derivatives **39-54**, which in turn were more active than the corresponding sulfanilamides **27-38**. This behavior has also been observed for the other three investigated isozymes [*96*]; (ii) some of the derivatives possessing bulky substitutents at the pyridinium ring (mainly phenyls, *tert*-butyls; *n*-butyl, *n*-propyl or *iso*-propyl), such as **34-37**, **51** and **67**, were very ineffective CA IX inhibitors, showing inhibition constants > 500 nM; (iii) another group of compounds, including **27**, **30-33**, **44**, and **60** showed a moderate inhibitory power towards the tumor-associated isozyme IX, showing K_I values in the range of 160 – 450 nM. Most of these compounds are sulfanilamide derivatives (except **44** and **60**), and the substitution pattern at the pyridinium ring includes (with one exception, **27**) at least one phenyl group in 4, or two phenyls in the 2 and 4 positions. It should be noted that the corresponding homosulfanilamides and 4-aminoethylbenzene-sulfonamides incorporating the same substitution pattern as the compounds mentioned above (sulfanilamides), lead to much better CA IX inhibitors (see later in the text); (iv) a third group of derivatives, including **38, 45-50, 52, 53, 61, 63-66, 68** and **69**, showed good CA IX inhibitory properties, with K_I values in the range of 64 – 135 nM. As mentioned above, except for the tetramethyl-pyridinium-substituted derivative **38**, most of these compounds incorporate 4-phenyl-pyridinium or 2,4-diphenylpyridinium moieties, whereas the group in position 6 is generally quite variable (alkyls or phenyl are tolerated). The most interesting observation regarding this subtype of CA IX inhibitors is constituted by the fact that the 2,4,6-triphenyl-pyridinium- and 2,6-

diphenyl-pyridinium derivatives of homosulfanilamide and 4-aminoethylbenzenesulfonamide (**52-53** and **68-69**) efficiently inhibit isozyme IX, although they act as very weak inhibitors for isozymes I, II and IV (Table 2). As it will be discussed shortly, this may be due to the fact that the hCA IX active site is larger than that of the other investigated isozymes, notably CA II, I and IV; (v) a last group of derivatives (**28-29; 39-43; 54; 55-59; 62** and **70**) showed very good CA IX inhibitory properties, these compounds possessing K_I values in the range of 6 – 54 nM, similarly to the clinically used inhibitors acetazolamide, methazolamide, dichlorophenamide and indisulam, for which the inhibition data are provided for comparison. It should be noted that three derivatives **58**, **59** and **70** showed inhibition constants < 10 nM, these being the most potent CA IX inhibitors ever reported up to now. Correlated with their membrane-impermeability [*96, 85*], it may be assumed that *in vivo* such compounds may lead for the first time to a selective CA IX inhibition. Thus, the best substitution pattern at the pyridinium ring includes either only compact alkyls (**39-41**, **54**, **55** and **70**), or 2,6-dialkyl-4-phenyl-pyridinium moieties (all compounds mentioned above except **62**, which incorporates a 2-methyl-4,6-diphenylpyridinium ring); (vi) the number of the substitutents at the pyridinium ring seems to be less important for the activity of this series of CAIs, since both di-, tri- or tetrasubstituted derivatives showed good inhibitory potency. The nature of these groups on the other hand – as discussed in detail above – is the most important parameter influencing CA inhibitory properties (together with the linker between the benzenesulfonamide moiety and the substituted pyridinium ring); (vii) the isozyme most similar to hCA IX regarding the affinity for these inhibitors was hCA II (which has 33 % homology with hCA IX) [Pastorek et al. (1994), supra] whereas the affinities of isozymes I and IV were rather different.

Screening of representative pyridinium derivatives of heterocyclic sulfonamides for inhibition of MN protein, and comparison with inhibition of other CA isozymes: *Isozyme I.* As seen from data of Table 3, all derivatives **71-91** reported here act as very efficient CAIs against this isozyme which is generally the most "resistant" to inhibitors of this type [*30, 31, 100, 102*]. Indeed, aminobenzolamide is already a highly potent CA I inhibitor (K_I of 6 nM), whereas inhibitors **71-91** show inhibition constants in the range of 3-12 nM, in contrast to the clinically used

sulfonamide CAIs which are much less effective inhibitors, with K_I values in the range of 30 - 1200 nM (Table 3). Thus, derivatives possessing several bulky groups (*i*-Pr; *t*-Bu; *n*-Pr; *n*-Bu; Ph, etc) substituting the pyridinium moiety, such as **73, 74, 77, 78, 82, 84, 85** showed a decreased inhibitory activity as compared to aminobenzolamide, with K_I values in the range of 7-12 nM (aminobenzolamide has a K_I of 6 nM against hCA I). The rest of the compounds were more efficient as compared to aminobenzolamide in inhibiting this isozyme, with K_I values in the range of 3-5 nM. Best CA I inhibitors were **75**, and **89-91** (K_I of 3 nM), all of which containing either only alkyl moieties or 4-Ph and other alkyl moieties substituting the pyridinium ring. These are probably the best CA I inhibitors ever reported up to now, since the clinically used CAIs show much higher inhibition constants against isozyme I (Table 3).

Isozyme II. Aminobenzolamide is already a very potent CA II inhibitor, with an inhibition constant around 2 nM. Several of the new inhibitors, such as **74,77,78,82-88** act as weaker CA II inhibitors as compared to aminobenzolamide, with K_I values in the range of 3.13-5.96 nM (but all these compounds act as potent inhibitors, being much more effective than the clinically used CAIs acetazolamide, methazolamide, dichlorophenamide or indisulam – see Table 3). Again the substitution pattern at the pyridinium ring is the main discriminator of activity for these compounds: all the less active derivatives mentioned above incorporate at least two bulky/long aliphatic groups; mainly in positions 2- and 6- of the pyridinium ring (*n*-Pr; *t*-Bu; *n*-Bu; and Ph). The best CA II inhibitors among derivatives **71-91** were those incorporating more compact 2,6-substituents at the pyridinium ring (such as Me, Et) together with a 4-Me or 4-Phe moiety, or those incorporating only aliphatic such groups, such as **71-73,75,76, 79-81, 89-91**, which showed K_I values in the range of 0.20-1.61 nM (thus, for the best inhibitors a factor of 10 increase in inhibitory power as compared to aminobenzolamide). It should be mentioned that *iso*-propyl-substituted compounds (**73, 79**) are active as CA II inhibitors, although their activity against CA I was not so good.

Isozyme IV. Most sulfonamides show inhibitory activity against CA IV intermediate between those towards CA I (less susceptible) and CA II (very high affinity for sulfonamides). This is also the trend observed with the sulfonamides

investigated here, derivatives of aminobenzolamide. Thus, the parent sulfonamide (shown in Figure 5) is a potent CA IV inhibitor, with a K_I value around 5 nM. The new derivatives of general formula (**B**) incorporating bulky pyridinium-ring substituents (such as **74, 77, 78, 82, 84-88, 90**) were less effective than aminobenzolamide, showing K_I values in the range of 5.2-10.3 nM, whereas the compounds showing the other substitution pattern mentioned above were better CA IV inhibitors, showing K_I values in the range of 2.0-4.7 nM.

Isozyme IX. Aminobenzolamide is less inhibitory against this isozyme (K_I of 38 nM) as compared to other isozymes discussed above. This behavior is difficult to explain at this point, since no X-ray crystal structure of this isozyme has been reported. A very encouraging result obtained with the new derivatives of general formula (**B**) reported here, was the observation that several of them show very high affinity for CA IX, with K_I values in the range of 3-9 nM (derivatives **71, 72, 75, 76**, and **89**). It may be seen that all of them incorporate aliphatic moieties (Me, Et and i-Pr) in positions 2- and 6- of the pyridinium ring, and either 4-Me or 4-Ph moieties. Only one compound is tetrasubstituted (**89**), again possessing only methyl moieties. The best CA IX inhibitor (and the best ever reported up to now) was **71**, which is almost 13 times more effective than benzolamide in inhibiting this isozyme. Another group of new derivatives, such as **73, 74, 77, 79, 80, 81, 83, 86-88, 90, 91,** showed effective CA IX inhibition, with K_I values in the range of 12-35 nM, being thus more effective than aminobenzolamide. They incorporate slightly bulkier groups as compared to the previously discussed ones. Again the less effective inhibitors (K_I values in the range of 40-43 nM) were those incorporating several bulky pyridinium substituents, such as **78, 84, 85** which contained either two *n*-Bu or one Ph and *n*-Bu/*t*-Bu in positions 2- and 6- of the pyridinium ring. Thus, SAR is now rather clear for this type of CAIs: best CA IX inhibitors should contain either only small, compact aliphatic moieties substituting the pyridinium ring, or they tolerate a 4-Ph moiety, but the 2,6-substituents should again be small, compact aliphatic moieties. In this particular case, 2,4,6-trisubstituted-pyridinium derivatives were more effective CA IX inhibitors as compared to the tetrasubstituted derivatives.

<u>Membrane impermeability of Heterocyclic Sulfonamide Inhibitors of CA IX</u>. As seen from data of Table 4 of Example 3, incubation of human red cells (which

contain high concentrations of isozymes I and II, i.e., 150 μM hCA I and 20 μM hCA II, but not the membrane-bound CA IV or CA IX) [*118*] with millimolar concentrations of different sulfonamide inhibitors, such as acetazolamide, or methazolamide, led to saturation of the two isozymes present in erythrocytes with inhibitor, already after short periods of incubation (30 min), whereas for benzolamide or aminobenzolamide, a similar effect is achieved after somehow longer periods (60 min) (Table 4). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide/aminobenzolamide with a pK_a of 3.2 for the second sulfonamido group [*58*] being present mainly as an (di)anion at the pH at which the experiment has been done (7.4), is already less diffusible and penetrates membranes in a longer time. Different cationic sulfonamides synthesized by us here, such as **71, 76, 89, 91,** in the same conditions, were detected only in very small amounts within the blood red cells, proving that they were unable to penetrate through the membranes, obviously due to their cationic nature. Even after incubation times as long as one hour (and longer, data not shown), only traces of such cationic sulfonamides were present inside the blood red cells, as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (Table 4). This demonstrates that the proposed approach for achieving membrane impermeability works well for the designed positively-charged sulfonamide CAIs of the general formula **(B)** (shown above), since the very small amount of sulfonamide detected may be due to contamination of the lysates with very small amount of membranes.

Design of Membrane-Impermeant Sulfonamide Inhibitors of CA IX

No X-ray crystal structure of isozyme IX is available up to now, in strong contrast with hCA II, for which many X-ray crystal structures are available (alone or in complexes with inhibitors and activators) [*1, 2, 14, 15, 19a, 19b, 37, 38*]. Examining the active site residues of these two isozymes and the architecture of hCA II, may help explain the above inhibition data and their relevance for CA IX specific inhibitors.

First of all, the zinc ligands and the proton shuttle residue of these two isozymes are identical [*33, 43, 72, 100, 101, 102, 114, 115, 117*]. An important

difference is constituted by the amino acid in position 131, which is Phe for hCA II and Val for hCA IX. Phe 131 is known to be very important for the binding of sulfonamide inhibitors to hCA II [*2, 46, 47*]: in many cases this bulky side chain limits the space available for the inhibitor aromatic moieties, or it may participate in stacking interactions with groups present in it (for recent examples see refs. [*2, 46, 47*]. Thus, the presence of a less bulky such residue in hCA IX (i.e., a valine) which is also unavailable for participation to stacking interactions has as a consequence the fact that the hCA IX active site is larger than that of hCA II. A second potentially important residue is 132, which is Gly in hCA II and Asp in hCA IX. This residue is situated on the rim of the hydrophilic half of the entrance to the active site of hCA II (and presumably also of hCA IX) and it is critical for the interaction with inhibitors possessing elongated molecules, as recenly shown by us [*19b*]. Strong hydrogen bonds involving the CONH moiety of Gly 132 were shown to stabilize the complex of this isozyme with a *p*-aminoethylbenzenesulfonamide derived inhibitor [*19b*]. In the case of hCA IX, the presence of aspartic acid in this position at the entrance of the active site may signify that: (i) stronger interactions with polar moieties of the inhibitor bound within the active site should be possible, since the COOH moiety possesses more donor atoms; (ii) this residue may have flexible conformations, fine-tuning in this way the interaction with inhibitors. Thus, the stronger hCA IX inhibition with some of these inhibitors (as compared to their affinity for isozyme II), such as for example **46-50**, **52, 53, 55, 58, 62** and **68-70**, might be explained just by the different interactions with the two active site residues mentioned above.

Therapeutic Use of MN-Specific Inhibitors

The MN-specific inhibitors of this invention, organic and/or inorganic, preferably organic, and as outlined above, may be used therapeutically in the treatment of neoplastic and/or pre-neoplastic disease, either alone or in combination with other chemotherapeutic drugs.

The MN-specific inhibitors can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable, non-toxic liquid vehicle.

Materials and Methods

General. Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400-4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ^{1}H-NMR spectra: Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); Elemental analysis: Carlo Erba Instrument CHNS Elemental Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Pyrylium salts were prepared by literature procedures, generally by olefin (or their precursors) bisacylation, as described in the literature [*6, 26, 108*], whereas aminobenzolamide as described earlier [*97*]. Other sulfonamides used as standards were commercially available.

General procedure for the preparation of compounds **71-91** (Pyridinium derivatives of aminobenzolamide)

An amount of 2.9 mM of aminobenzolamide [*97*] and 2.9 mM of pyrylium salt II (depicted in Figure 5) were suspended in 5 mL of anhydrous methanol and poured into a stirred mixture of 14.5 mM of triethylamine and 5.8 mM of acetic anhydride. After five minutes of stirring, another 10 mL of methanol were added to the reaction mixture, which was heated to reflux for 15 min. Then 14.5 mM of acetic acid was added and heating was continued for 2-5 hours. The role of the acetic anhydride is to react with the water formed during the condensation reaction between the pyrylium salt and the aromatic amine, in order to shift the equilibrium towards the formation of the pyridinium salts of the general formula (**B**) (shown above). In the case of aminobenzolamide, this procedure is the only one which gave acceptable yields in pyridinium salts, probably due to the deactivating effect of the sulfamoylaminothiadiazole moiety on the amine group, which becomes poorly nucleophilic and unreactive towards these reagents. The precipitated pyridinium salts obtained were purified by treatment with concentrated ammonia solution (which also converts the eventually unreacted pyrylium salt to the corresponding pyridine which is soluble in acidic medium), reprecipitation with perchloric acid and recrystallization from water with 2-5 % $HClO_4$.

Purification of Catalytic Domain of CA IX

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al. [*72*]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2 % Triton X-100, as described by Wingo et al. [*116*]. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10mM Hepes (pH 7.5), 10mM Tris HCl, 100mM Na_2SO_4 and 1mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate [*44*]. Optionally, the protein was further purified by sulfonamide affinity chromatography [*44*], the amount of enzyme was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate [*44*].

CA I, II and IV purification

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group [*54*]. Cell growth conditions were those described in ref. [*12*], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [*45*]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}.cm^{-1}$ for CA I and 54 $mM^{-1}.cm^{-1}$ for CA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively [*53, 84*]. CA IV was isolated from bovine lung microsomes as described

by Maren et al, and its concentration has been determined by titration with ethoxzolamide [*59*].

Enzyme assays

CA CO2 Hydrase Activity Assay

An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA CO_2 hydration activity assays [*44*]. A stopped flow variant of the Poker and Stone spectrophotometric method [*76*] has been employed, using an SX.18MV-R Applied Photophysics stopped flow instrument, as described previously [*43*]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate [*44*]. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.

CA Esterase Activity Assay

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [*76*]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2.10^{-2} and 1.10^{-6} M, working at 25°C. A molar absorption coefficient ε of 18,400 $M^{-1}.cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature [*76*]. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported

throughout the paper are the mean of such results. Stock solutions of inhibitor (1-3 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constant K_I was determined as described in references [*44, 76*].

<u>Membrane Permeance Assay:</u>
<u>Ex vivo Penetration through Red Blood Cells</u>

An amount of 10 mL of freshly isolated human red cells thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min were treated with 25 mL of a 2 mM solution of sulfonamide inhibitor. Incubation has been done at 37 °C with gentle stirring, for periods of 30 - 120 min. After the incubation times of 30, 60 and 120 min., respectively, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer, in order to eliminate all unbound inhibitor [*81, 96, 98*]. The cells were then lysed in 25 mL of distilled water, centrifuged for eliminating membranes and other insoluble impurities. The obtained solution was heated at 100 °C for 5 minutes (in order to denature CA-s) and sulfonamides possibly present have been assayed in each sample by three methods: a HPLC method [*36*]; spectrophotometrically [*4*] and enzymatically [*76*].

<u>*HPLC*</u>: A variant of the methods of Gomaa [*36*] has been developed by us, as follows: a commercially available 5 μm Bondapak C-18 column was used for the separation, with a mobile phase made of acetonitrile – methanol – phosphate buffer (pH 7.4) 10:2:88 (v/v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulphadiazine (Sigma) as internal standard. The retention times were: 12.69 min for acetazolamide; 4.55 min for sulphadiazine; 10.54 min for benzolamide; 12.32 min for aminobenzolamide; 3.15 min for **71**; 4.41 min for **76**; 3.54 min for **89**; and 4.24 min for **91**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide, and wavelength in the range of 270 – 310 nm in the case of the other sulfonamides.

Spectrophotometrically: A variant of the pH-induced spectrophotometric assay of Abdine et al. [*4*] has been used, working for instance at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, for sulfanilamide, etc. Standardized solutions of each inhibitor have been prepared in the same buffer as the one used for the membrane penetrability experiments.

Enzymatically: the amount of sulfonamide present in the lysate has been evaluated based on hCA II inhibition measured with the esterase method, as described above [*76*]. Standard inhibition curves have been obtained previously for each sulfonamide, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate. Mention should be made that the three methods presented above led to results in good agreement, within the limits of the experimental errors.

Statistical analysis: Values are expressed ± standard error of measurement. Statistical significance was determined using an unpaired t-test with $p<0.05$ considered significant.

The following examples are for purposes of illustration only and are not meant to limit the invention in any way.

Example 1

Inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides

The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of aromatic and heterocyclic sulfonamides, including the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison.

Chemistry. Sulfonamides investigated for the inhibition of the tumor-associated isozyme CA IX, of types **1-26** are shown in Figure 4A-B. Compounds **1-6**, **11-12**, **20** and **26** are commercially available, whereas **7-10** [*43*], **13-19** [*24, 79, 90, 97*] and **21-25** [*79*] were prepared as reported earlier. The six clinically used

compounds were also assayed, since no such data are available in the literature.

CA inhibition data. Inhibition data against four CA isozymes, CA I, II, IV and IX [*44, 72, 116*], with the above mentioned compounds **1-26** and the six clinically used inhibitors, are shown in Table 1.

Table 1: CA I, II, IV and IX inhibition data with sulfonamides **1-26** and clinically used inhibitors.

Inhibitor	K_I* (nM)			
	hAC I[a]	hCA II[a]	bCA IV[b]	hCA IX[c]
1	45400	295	1310	33
2	25000	240	2200	238
3	28000	300	3000	294
4	78500	320	3215	305
5	25000	170	2800	103
6	21000	160	2450	33
7	8300	60	180	245
8	9800	110	320	264
9	6500	40	66	269
10	6000	70	125	285
11	5800	63	154	24
12	8400	75	160	39
13	8600	60	540	41
14	9300	19	355	30
15	6	2	5	38
16	164	46	129	34
17	185	50	144	20
18	109	33	72	31
19	95	30	72	24
20	690	12	154	16
21	55	8	17	14
22	21000	125	415	32

(Table 1, continued)

23	23000	133	438	30
24	24000	125	560	21
25	18000	110	450	22
26	135	40	86	26
AAZ	250	12	70	25
MZA	50	14	36	27
EZA	25	8	13	34
DCP	1200	38	380	50
DZA	50000	9	43	52
BRZ	-	3	45	37

[a] Human cloned isozymes, esterase assay method [*76*];
[b] Isolated from bovine lung microsomes, esterase assay method [*76*];
[c] Human cloned isozyme, CO_2 hydrase assay method [*44, 72, 116*].

We report here the first inhibition study of the tumor-associated, transmembrane isozyme CA IX with a series of aromatic and heterocyclic sulfonamides, including also the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) are also provided for comparison. Very interesting inhibition profile against CA IX with these sulfonamides has been detected, which is a promising discovery for the potential design of CA IX-specific inhibitors, with applications as antitumor agents. Several nanomolar CA IX inhibitors have been detected, both among the aromatic (such as orthanilamide, homosulfanilamide, 4-carboxy-benzenesulfonamide, 1-naphthalene- sulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocyclic (such as 1,3,4-thiadiazole-2-sulfonamide, benzothiazole-2-sulfonamide, etc.) sulfonamides investigated.

Example 2
The first selective, membrane-impermeant inhibitors targeting the tumor-associated isozyme IX

Up to now no CA IX inhibition studies with this type of membrane-impermeant CAIs have been reported. Thus, we decided to explore some of the pyridinium derivatives of general formula **(A)** for their interaction with the catalytic domain of tumor-associated isozyme IX, recently cloned and purified by the inventors [*33, 43, 114, 115, 117*], as well as the cytosolic, physiologically relevant isozymes CA I, II and the membrane-anchored isozyme CA IV [*88, 96*].

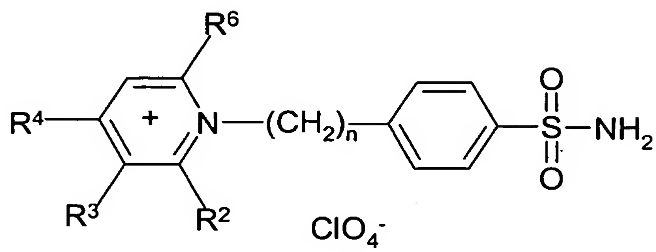
The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of positively-charged, pyridinium derivatives of sulfanilamide, homosulfanilamide and 4-aminoethyl-benzenesulfonamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison. This is the first report of inhibitors that may selectively target CA IX, due to their membrane-impermeability and high affinity for this clinically relevant isozyme.

CA inhibition.

Data of Table 2 clearly show that most of the compounds **27-70** act as efficient CA IX inhibitors, and that their affinity for this isozyme differs considerably as compared to affinities for the cytosolic isozymes CA I and II, and the other membrane-associated isozyme investigated, CA IV.

In a series of substituted-pyridinium derived sulfanilamides, homosulfanilamides and *p*-aminoethylbenzenesulfonamides, a large number of effective hCA IX inhibtors were detected. Some low nanomolar CA IX inhibitors were reported for the first time. Since these compounds are membrane-impermeant due to their salt-like character, and as hCA IX is present on the extracellular side of many tumors with poor clinical prognosis, compounds of this type target specifically this tumor-associate CA isozyme without affecting the cytosolic CAs known to play important physiological functions. Thus, compounds of this type may constitute the basis of new anticancer therapies based on CA inhibitors.

Table 2: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **27-70**.



A

Compound	R^2	R^3	R^4	R^6	K_I *			
					hCA I[a] (μM)	hCA II[a] (nM)	bCA IV[b] (nM)	hCA IX[c] (nM)
27	Me	H	Me	Me	10	150	290	165
28	Me	H	Ph	Me	7	60	211	48
29	Et	H	Ph	Et	6	60	182	43
30	*n*-Pr	H	Ph	*n*-Pr	10	120	194	178
31	*i*-Pr	H	Ph	*i*-Pr	5	50	90	160
32	Me	H	Ph	Ph	40	210	852	280
33	Et	H	Ph	Ph	43	400	1300	450
34	*n*-Pr	H	Ph	Ph	140	580	1483	>500
35	*i*-Pr	H	Ph	Ph	125	440	2102	>500
36	*n*-Bu	H	Ph	Ph	305	620	2155	>500
37	Ph	H	Ph	Ph	290	510	2500	>500
38	Me	Me	Me	Me	5	40	61	72
39	Me	H	Me	Me	7	50	92	38
40	*i*-Pr	H	Me	Me	6	50	80	42
41	*i*-Pr	H	Me	*i*-Pr	11	80	144	54
42	Me	H	Ph	Me	4	20	70	26
43	Et	H	Ph	Et	2	21	52	29
44	*n*-Pr	H	Ph	*n*-Pr	24	90	163	230
45	*i*-Pr	H	Ph	*i*-Pr	12	61	101	100
46	Me	H	Ph	Ph	32	121	161	64
47	Et	H	Ph	Ph	42	314	983	79

(Table 2, continued)

48	*n*-Pr	H	Ph	Ph	130	390	1260	85
49	*i*-Pr	H	Ph	Ph	112	370	1214	80
50	*n*-Bu	H	Ph	Ph	300	595	2104	135
51	*t*-Bu	H	Ph	Ph	110	321	1070	>500
52	Ph	H	Ph	Ph	280	472	1956	120
53	Ph	H	H	Ph	280	493	1954	106
54	Me	Me	Me	Me	3	30	51	35
55	Me	H	Me	Me	4	21	60	14
56	*i*-Pr	H	Me	Me	2	15	32	31
57	*i*-Pr	H	Me	*i*-Pr	3	20	70	49
58	Me	H	Ph	Me	1	8	20	6
59	Et	H	Ph	Et	1	9	21	8
60	*n*-Pr	H	Ph	*n*-Pr	7	42	82	205
61	*i*-Pr	H	Ph	*i*-Pr	6	21	70	89
62	Me	H	Ph	Ph	18	103	144	37
63	Et	H	Ph	Ph	40	220	761	70
64	*n*-Pr	H	Ph	Ph	112	270	1055	84
65	*i*-Pr	H	Ph	Ph	94	350	864	78
66	*n*-Bu	H	Ph	Ph	290	544	2008	120
67	*t*-Bu	H	Ph	Ph	92	275	1000	>500
68	Ph	H	Ph	Ph	270	419	1830	95
69	Ph	H	H	Ph	265	420	1905	81
70	Me	Me	Me	Me	2	10	21	8
acetazolamide					0.25	12	70	25
methazolamide					0.05	14	36	27
dichlorophenamide					1.2	38	380	50
indisulam					0.03	15	65	24

[a] Human (cloned) isozymes; [b] From bovine lung microsomes; [c] Catalytic

domain of the human, cloned isozyme.

* errors in the range of ± 10 % of the reported value, from three different determinations.

For compunds 27-38: n = 0; **39-54**: n = 1; **55-70**: n = 2

Example 3

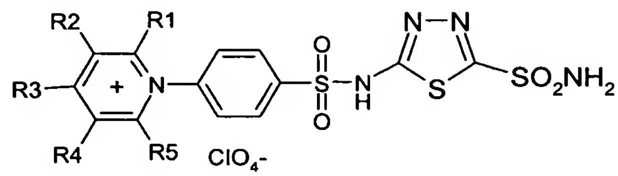
Design of selective, membrane-impermeant heterocyclic sulphonamide inhibitors targeting the human tumor-associated isozyme IX

A series of positively-charged sulfonamides were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrilium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring. These new compounds are membrane-impermeant due to their salt-like character and were assayed for the inhibition of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, the membrane-anchored bCA IV and the membrane-bound, tumor associated isozyme hCA IX. The high affinity of these new derivatives for the tumor-associated isozyme CA IX and their membrane impermeability, make this type of CA inhibitors interesting candidates for the selective inhibition of only the tumor associated isozyme and not the cytosolic ones, for which they also show high potency.

Results

CA inhibition. Inhibition data against isozymes I, II, IV and IX with compounds **71-91** reported here are shown in Table 3.

Table 3: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **71-91**.



B

	R^1	R^2	R^3	R^4	R^5	K_I *(nM) hCA I[a]	hCA II[a]	bCA IV[b]	hCA IX[c]
71	Me	H	Me	H	Me	4	0.26	2.1	3
72	*i*-Pr	H	Me	H	Me	4	0.39	3.0	5
73	*i*-Pr	H	Me	H	*i*-Pr	7	1.54	4.7	16
74	*t*-Bu	H	Me	H	*t*-Bu	11	3.13	9.4	34
75	Me	H	Ph	H	Me	3	0.20	2.0	6
76	Et	H	Ph	H	Et	4	0.21	2.3	9
77	*n*-Pr	H	Ph	H	*n*-Pr	9	3.45	8.1	35
78	*n*-Bu	H	Ph	H	*n*-Bu	10	4.62	10.3	40
79	*i*-Pr	H	Ph	H	*i*-Pr	5	1.61	4.1	30
80	Me	H	Ph	H	Ph	4	1.21	3.0	24
81	Et	H	Ph	H	Ph	5	1.14	3.8	29
82	*n*-Pr	H	Ph	H	Ph	8	3.90	6.0	40
83	*i*-Pr	H	Ph	H	Ph	6	3.74	4.5	32
84	*n*-Bu	H	Ph	H	Ph	8	4.95	8.4	45
85	*t*-Bu	H	Ph	H	Ph	12	4.11	7.0	43
86	Ph	H	Me	H	Ph	6	4.78	5.8	12
87	Ph	H	Ph	H	Ph	5	5.96	5.6	12
88	Ph	H	H	H	Ph	5	4.93	5.4	16
89	Me	Me	Me	H	Me	3	0.30	2.4	5
90	Me	Me	Ph	H	Me	3	1.24	5.2	15
91	Me	R^3,R^5= $(CH_2)_9$; R^4=Me			Me	3	1.37	4.6	12

(Table 3, continued)

aminobenzolamide	6	2.04	5.1	38
acetazolamide	250	12	70	25
methazolamide	50	14	36	27
dichlorophenamide	1200	38	380	50
indisulam	30	15	65	24

[a] Human (cloned) isozymes, esterase assay method [*76*].

[b] From bovine lung microsomes, esterase assay method [*76*].

[c] Catalytic domain of the human, cloned isozyme, CO_2 hydrase assay method *[44]*.

* Errors in the range of ± 10 % of the reported value, from three different determinations.

Ex vivo penetration through red blood cells. Levels of sulfonamides in red blood cells after incubation of human erythrocytes with millimolar solutions of inhibitor for 30-60 min (both classical as well as positively-charged sulfonamides were used in such experiments) are shown in Table 4 [*4, 12, 36, 45, 53, 54, 58, 59, 84, 116, 118*].

Table 4: Levels of sulfonamide CA inhibitors (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide has been determined by three methods: HPLC; electronic spectroscopy (ES) and the enzymatic method (EI) – see Experimental for details.

Inhibitor	[sulfonamide], μM*					
	t = 30 min			t = 60 min		
	HPLC[a]	ES[b]	EI[c]	HPLC[a]	ES[b]	EI[c]
AAZ	136	139	140	160	167	163
MZA	170	169	165	168	168	167
Benzolamide	110	108	112	148	146	149
Aminobenzolamide	125	127	122	154	156	158
71	0.3	0.5	0.5	0.4	0.5	0.3
76	1.0	1.1	1.0	1.1	1.2	1.1
89	0.3	0.2	0.5	0.3	0.6	0.4
91	0.4	0.3	0.5	0.3	0.6	0.5

* Standard error (from 3 determinations) < 5 % by : [a] the HPLC method [*36*]; [b] the electronic spectroscopic method [*4*]; [c] the enzymatic method [*76*].

The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within ± 0.4 % of the theoretical values; IR and NMR spectroscopy) that confirmed their structure (see Materials and Methods and Table 5 below for details) and were assayed for the inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX.

Table 5: Elemental analysis data for the compounds described in Example 3

No	Formula	Elemental analysis data (calc./found)		
		% C	% H	% N
71	$C_{16}H_{18}N_5O_4S_3^+ ClO_4^-$	35.59/35.32	3.36/3.62	12.97/12.93
72	$C_{18}H_{22}N_5O_4S_3^+ ClO_4^-$	38.06/37.95	3.90/4.16	12.33/12.18
73	$C_{20}H_{26}N_5O_4S_3^+ ClO_4^-$	40.30/39.99	4.40/4.54	11.75/11.63
74	$C_{22}H_{30}N_5O_4S_3^+ ClO_4^-$	42.34/42.56	4.84/4.76	11.22/11.03
75	$C_{21}H_{20}N_5O_4S_3^+ ClO_4^-$	41.89/42.02	3.35/3.03	11.63/11.48
76	$C_{23}H_{24}N_5O_4S_3^+ ClO_4^-$	43.84/43.88	3.84/3.62	11.11/10.95
77	$C_{25}H_{28}N_5O_4S_3^+ ClO_4^-$	45.62/45.60	4.29/4.36	10.64/10.50
78	$C_{27}H_{32}N_5O_4S_3^+ ClO_4^-$	47.26/47.45	4.70/4.89	10.21/10.14
79	$C_{25}H_{28}N_5O_4S_3^+ ClO_4^-$	45.62/45.49	4.29/4.18	10.64/10.61
80	$C_{26}H_{22}N_5O_4S_3^+ ClO_4^-$	47.02/46.79	3.34/3.33	10.55/10.23
81	$C_{27}H_{24}N_5O_4S_3^+ ClO_4^-$	47.82/47.73	3.57/3.73	10.33/10.40
82	$C_{28}H_{26}N_5O_4S_3^+ ClO_4^-$	48.59/48.83	3.79/3.91	10.12/10.24
83	$C_{28}H_{26}N_5O_4S_3^+ ClO_4^-$	48.59/48.27	3.79/3.82	10.12/10.05
84	$C_{29}H_{28}N_5O_4S_3^+ ClO_4^-$	49.32/49.59	4.00/4.23	9.92/9.67
85	$C_{29}H_{28}N_5O_4S_3^+ ClO_4^-$	49.32/49.16	4.00/3.94	9.92/9.71
86	$C_{26}H_{22}N_5O_4S_3^+ ClO_4^-$	47.02/47.25	3.34/3.18	10.55/10.46
87	$C_{31}H_{24}N_5O_4S_3^+ ClO_4^-$	51.27/51.50	3.33/3.60	9.64/9.67
88	$C_{25}H_{20}N_5O_4S_3^+ ClO_4^-$	46.19/46.28	3.10/2.95	10.77/10.67
89	$C_{17}H_{20}N_5O_4S_3^+ ClO_4^-$	36.86/36.72	3.64/3.53	12.64/12.45
90	$C_{22}H_{22}N_5O_4S_3^+ ClO_4^-$	42.89/42.70	3.60/3.84	11.37/11.15
91	$C_{24}H_{32}N_5O_4S_3^+ ClO_4^-$	44.34/44.57	4.96/4.99	10.77/10.51

Conclusions

We report here a general approach for the preparation of positively-charged, membrane-impermeant sulfonamide CA inhibitors with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound ones CA IV and CA IX. They were obtained by attaching substituted-pyridinium moieties to

aminobenzolamide, a very potent CA inhibitor itself. Ex vivo studies showed the new class of inhibitors reported here to discriminate for the membrane-bound versus the cytosolic isozymes. Correlated with the low nanomolar affinity of some of these compounds for the tumor-associated isozyme CA IX, this report constitutes the basis of selectively inhibiting only the target, tumor-associated CA IX in vivo, whereas the cytosolic isozymes would remain unaffected.

Characterization of Compounds 71-91 (For preparation, see Materials and Methods Section)

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-trimethyl-pyridinium perchlorate **71**: white crystals, mp >300°C; IR (KBr), cm^{-1} (bands in italics are due to the anion): 595, *625*, 664, 787, 803, 884, 915, *1100*, 1150, 1190, 1200, 1285, 1360, 1495, 1604, 3065; ^{1}H-NMR (D_2O), δ, ppm: 3.08 (s, 6H, 2,6-Me_2); 3.11 (s, 3H, 4-Me), 7.30 - 8.06 (m, AA'BB',4H, ArH from phenylene); 9.05 (s,2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal $C_{16}H_{18}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-dimethylpyridinium perchlorate **72**, colorless crystals, mp 29o-1°C; IR (KBr), cm^{-1} : 625, 680, 720, 1100, 1165, 1330, 1640, 3020, 3235; ^{1}H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr); 2.80 (s, 3H, 6-Me); 2.90 (s, 3H, 4-Me); 3.49 (heptet, 1H, CH from *i*-Pr); 7.25 - 8.43 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal $C_{18}H_{22}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-iso-propyl-4-methylpyridinium perchlorate **73**, tan crystals, mp 278-9°C; IR (KBr), cm^{-1} : 625, 685, 820, 1100, 1165, 1340, 1635, 3030, 3250; ^{1}H-NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr); 2.83 (s, 3H, 4-Me); 3.42 (heptet, 2H, 2CH from 2 *i*-Pr); 7.31 - 8.51 (m, AA'BB', 4H, ArH from 1,4-phenylene); 8.05 (s, 2H, ArH, 3,5-H from pyridinium). Anal $C_{20}H_{26}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-dimethyl-4-phenylpyridinium perchlorate **75**, white crystals, mp > 300 °C; IR

(KBr), cm^{-1} : 625, 690, 770, 1100, 1170, 1330, 1635, 3030, 3260, 3330; ^{1}H-NMR (TFA), δ, ppm: 2.62 (s, 6H, 2,6-$(Me)_2$); 8.10 - 9.12 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $C_{21}H_{20}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diethyl-4-phenylpyridinium perchlorate **76**, tan crystals, mp 267-8 °C; IR (KBr), cm^{-1} : 625, 695, 765, 1100, 1180, 1340, 1630, 3040, 3270, 3360; ^{1}H-NMR (TFA), δ, ppm: 1.43 (t, 6H, 2 Me from ethyl); 2.82 (q, 4H, 2 CH_2 from Et); 7.68 - 8.87 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $C_{23}H_{24}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-n-propyl-4-phenylpyridinium perchlorate **77**, colorless crystals, mp 235-7 °C ; IR (KBr), cm^{-1} : 625, 695, 770, 1100, 1180, 1340, 1630, 3050, 3220, 3315; ^{1}H-NMR (TFA), δ, ppm: 1.06 (t, 6H, 2 Me from propyl); 1.73 (sextet, 4H, 2CH_2 (β) from *n*-Pr); 2.84 (t, 4H, 2 CH_2 (α) from *n*-Pr); 7.55 - 8.71 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $C_{25}H_{28}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-isopropyl-4-phenylpyridinium perchlorate **79**, white crystals, mp 278-9 °C; IR (KBr), cm^{-1} : 625, 690, 765, 1100, 1180, 1340, 1625, 3040, 3270, 3315; ^{1}H-NMR (TFA), δ, ppm: 1.45 (d, 12H, 4 Me from *i*-Pr); 2.95 (heptet, 2H, 2 CH from *i*-Pr); 7.92 - 8.97 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $C_{25}H_{28}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-methyl-4,6-diphenylpyridinium perchlorate **80**, white crystals, mp 298-99 °C; IR (KBr), cm^{-1} : 625, 710, 770, 1100, 1170, 1345, 1625, 3040, 3245, 3350; ^{1}H-NMR (TFA), δ, ppm: 2.75 (s, 3H, 2-Me); 7.53 - 8.70 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal $C_{26}H_{22}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-ethyl-4,6-diphenylpyridinium perchlorate **81**, white crystals, mp 254-5 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1180, 1340, 1620, 3040, 3250, 3350; ^{1}H-NMR (TFA), δ, ppm: 1.52 (t, 3H, Me from ethyl); 2.97 (q, 2H, CH_2); 7.40 - 8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal $C_{27}H_{24}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-n-propyl-4,6-diphenylpyridinium perchlorate **82**, white crystals, mp 214-5 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1180, 1340, 1620, 3030, 3270, 3350; ^{1}H-NMR (TFA), δ, ppm: 1.03 (t, 3H, Me from propyl); 1.95 (sextet, 2H, β-CH_2 from *n*-Pr); 2.88 (t, 2H, α-CH_2 from *n*-Pr); 7.39 - 8.55 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal $C_{28}H_{26}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-diphenylpyridinium perchlorate **83**, white crystals, mp 186-8 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1170, 1340, 1620, 3040, 3250, 3360; ^{1}H-NMR (TFA), δ, ppm: 1.51 (d, 6H, 2 Me from *i*-propyl); 2.50 - 3.27 (m, 1H, CH from *i*-Pr); 7.32 - 8.54 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal $C_{28}H_{26}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-n-butyl-4,6-diphenylpyridinium perchlorate **84**, white crystals, mp 241-3 °C; IR (KBr), cm^{-1} : 625, 710, 770, 1100, 1180, 1335, 1625, 3040, 3260, 3345; ^{1}H-NMR (TFA), δ, ppm: 0.93 (t, 3H, Me from butyl); 1.12 - 2.14 (m, 4H, CH_3-*CH_2-CH_2*-CH_2 from *n*-Bu); 2.96 (t, 2H, α-CH_2 from *n*-Bu); 7.21 - 8.50 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal $C_{29}H_{28}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-tert-butyl-4,6-diphenylpyridinium perchlorate **85**, white crystals, mp 203-5°C; IR (KBr), cm^{-1} : 625, 705, 765, 1100, 1160, 1310, 1620, 3060, 3270; ^{1}H-NMR (TFA), δ, ppm: 1.91 (s, 9H, *t*-Bu); 6.80 - 8.74 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal $C_{29}H_{28}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-triphenhyl-pyridinium perchlorate **87** : pale yellow crystals, mp>300°C; IR (KBr), cm^{-1} (bands in italics are due to the anion): *625*, 635, 703, 785, 896, *1100*, 1150, 1204, 1355, 1410, 1520, 1600, 3065; ^{1}H-NMR (D_2O), δ, ppm: 7.50-8.60 (m, 19H, ArH, 3Ph + C_6H_4); 9.27 (s,2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal $C_{31}H_{24}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diphenylpyridinium perchlorate **88**, yellow crystals, mp 218-20°C; IR (KBr),

cm^{-1} : 625, 705, 765, 1100, 1160, 1335, 1615, 3050, 3260; ^1H-NMR (TFA), δ, ppm: 6.75 - 8.43 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal $C_{25}H_{20}N_5O_4S_3^+$ ClO_4^- (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,3,4,6-tetramethylpyridinium perchlorate **89**, tan crystals, mp > 300°C; IR (KBr), cm^{-1} : 625, 800, 1100, 1165, 1330, 1630, 3030, 3305; ^1H-NMR (TFA), δ, ppm: 2.62 (s, 3H, 4-Me); 2.74 (s, 3H, 3-Me); 2.88 (s, 6H, 2,6-$(Me)_2$); 7.21 - 8.50 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.93 (s, 1H, ArH, 5-H from pyridinium). Anal $C_{17}H_{20}N_5O_4S_3^+$ ClO_4^- (C, H, N).

The description of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical application to enable thereby others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

All references cited herein are hereby incorporated by reference.